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ANNUAL PROGRESS REPORT - FY 1976.(U)
JUL 76 F B ABELES, A O ANDERSON, J B ARENSMAN

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PROGRESS REPORT
FY 1976

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UNITED STATES ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

Frederick, Maryland 21701

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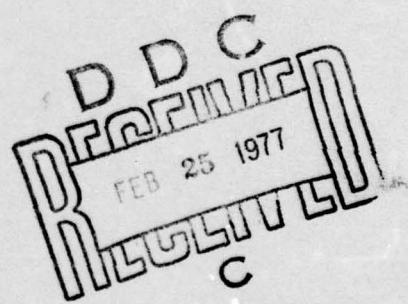
U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
FREDERICK, MARYLAND 21701

ANNUAL PROGRESS REPORT

FISCAL YEAR 1976

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Project 3A762760A834

1 July 1976

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A report of progress on the research program of the U. S. Army Medical Research-Institute of Infectious Diseases on Medical Defense Against Biological Agents (U) for Fiscal Year 1976 is presented.			

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FOREWORD

This report

This FY 1976 Annual Progress Report is a general review of research activities of the U. S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland, conducted under Project 3A762760A834, Medical Defense Against Biological Agents (U) and a small effort under the In-House Laboratory Independent Research Program (ILIR). The following subject areas are included:

- 3A762760A834 01 Pathogenesis of Infection of Military Importance;
- 3A762760A834 02 - Prevention and Treatment of Biological Agent Casualties; and
- 3A762760A834 03 - Laboratory Identification of Biological Agents.

Nine contracts were in effect with educational institutions or industrial firms. Reports are available through Defense Documentation Center.

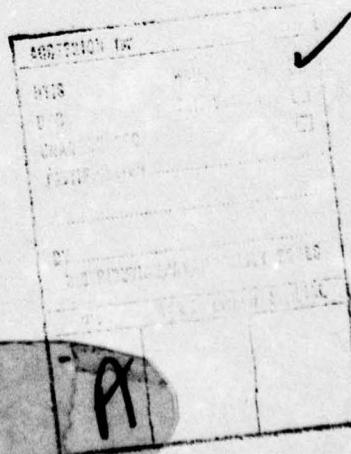
Tasks of the basic project are subdivided into work units, each identified by task number plus a 3-digit suffix. Subdivisions are identified in accordance with the following scheme:

General	001-099
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Rickettsial diseases	300-399
Viral diseases	400-699
Mycotic diseases	700-799
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In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council. The facilities are fully accredited by the American Association of Accreditation of Laboratory Animal Care.

The period 7T will be included in Fiscal Year 1977 Annual Progress Report.

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** Terminated.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL
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10. NO./CODES: 62760A	PROGRAM ELEMENT PROJECT NUMBER 3A762760A834	11. WORK UNIT NUMBER 003				
11. PRIMARY B. CONTRIBUTING	C. CONFIDENTIAL CARDS 114(e)(f)					
12. TITLE (Provide with Security Classification Code) (U) Tissue enzyme changes in infectious disease of military medical importance						
13. SCIENTIFIC AND TECHNOLOGICAL AREA ^e 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry						
14. START DATE 64 10	15. ESTIMATED COMPLETION DATE CONT	16. FUNDING AGENCY DA	17. PERFORMANCE METHOD C. In-house			
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23. KIND OF AWARD: F. CUM. AMT.		24. PERFORMING ORGANIZATION				
25. RESPONSIBLE DOD ORGANIZATION NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		NAME: Physical Sciences Division USAMRIID ADDRESS: Fort Detrick, MD 21701				
26. RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Furnish DSN if U.S. Academic Institution) NAME: Petrella, V. J. TELEPHONE: 301 663-7181 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: George, D. T. NAME:				
27. GENERAL USE Foreign intelligence considered		POC:DA				
28. KEY WORDS (Provide each with Security Classification Code) (U) Cyclic AMP; (U) Adenylate cyclase; (U) Electron paramagnetic resonance (EPR); (U) Triglycerides; (U) Cholera toxin; (U) Military medicine; BW Defense						
29. TECHNICAL OBJECTIVE, 30. APPROACH, 31. PROGRESS (Furnish individual paragraphs identified by number. Provide text of each with Security Classification Code.)						
23. (U) To study serial changes in tissue enzyme systems during the course of experimental infections in an attempt to uncover changes that might have diagnostic or therapeutic use for medical defense against BW agents.						
24. (U) Studies of the adenylate cyclase system are conducted to determine what effect infections had on this endocrine-metabolic parameter. Further studies may include this system in the evaluation of the effects of infection on the lipolytic activity of the isolated fat body.						
25. (U) 75 07 - 76 06 - To better understand the mechanism of action of cholera toxin (CT) EPR studies were conducted on intact thymocytes during the time lag which occurs between CT binding and subsequent adenylate cyclase activation. Although revealing no clear alteration in membrane fluidity, these studies contributed to a better understanding of the position and environment of the membrane-bound enzyme, adenylate cyclase. Results illustrated the presence of multilipid domains and the importance of selected lipids and lipid-protein interactions in the adenylate cyclase-cyclic AMP system. The EPR studies terminate the CT work by this investigator. A proposal has been submitted wherein mechanisms by which alterations in tissue enzymes responsible for maintenance of substrate (free fatty acids) for ketone bodies and serum triglycerides could be studied. Concentrations of these 2 moieties are altered by infection; results are inconclusive at this time.						
With publication of a paper, the study is complete. Publications: Biochim. Biophys Acta 421:237-245, 1976. Clin. Res. 24:373A, 1976.						
* Available to contractors upon originator's approval.						

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Aspects of Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 003: Tissue Enzyme Changes in Infectious Disease of Military Medical Importance

Background:

Part I.

The action of Vibrio cholerae in the small intestine is the result of an enterotoxin, cholera toxin (CT), elaborated by this organism.¹ The massive fluid and electrolyte losses which occur in clinical cholera infection are the result of CT-stimulation of intestinal adenylyl cyclase (AC). In addition to its effect on intestinal cells, CT has also been shown to increase AC activity in a number of tissues including thymocytes. Although certain physical characteristics of the enterotoxin such as its structure and binding to membrane have been well defined, the mechanism by which CT, once bound to receptor, stimulates AC has not been established. Of special concern in this regard is the lag phase required for activation of the enzyme once the toxin is bound. In order to study this lag phase more carefully, electron paramagnetic resonance (EPR) techniques were applied in the hope of noting some characteristic changes in membrane fluidity. Speculation as to the mode of action of CT includes theories of internalization and/or lateral diffusion, and such events may be visible through EPR.

Part II.

Although many studies have reported hypertriglyceridemia during endotoxemia or gram negative sepsis in man and experimental animals,²⁻⁴ only recently have attempts been made to describe mechanistically this phenomenon. From the standpoint of the infected organism, such information is vital if any rational attempt to support the energy needs of the infected host is to come. The energy needs of the host are of great concern, since during acute infectious illness, virtually every biochemical pathway examined thus far becomes involved. Changes in these pathways follow from a number of defensive reactions, some of which are catabolic, exemplified by losses in major intracellular elements or negative nitrogen balance. It is the greater need for metabolizable energy that the host organism must satisfy; only through careful studies of intermediary metabolism can proper approaches to supportive therapy be devised.

The fact that hypertriglyceridemia and depressed hepatic ketone body⁵ concentrations can coexist during bacterial infections raises some fundamental questions. Where do the triglycerides come from in the fasted infected state? Most reports suggest synthesis by the liver utilizing serum free fatty acids (FFA). The major fasting source of FFA would have to be the fat depots. If fatty acid levels in the serum are high enough to support triglyceride synthesis, why isn't the liver producing ketone bodies, a ready form of energy to an organism which is depleting structural protein for energy?

The objective of this investigation is to test possible mechanisms by which alterations in the intermediary metabolism of lipids can occur during infection. Emphasis will be placed on studies which could account for observed elevated triglycerides as well as lowered ketone body formation. This will be accomplished by the study of those enzymes responsible for maintenance of normal concentrations of these moieties in the organism. Particular attention will be paid to the lipolytic processes of the fat depot which maintain levels of substrate (FFA) for triglycerides and ketone body production. Post-heparin lipolytic activity (PHLA), which clears triglycerides from the serum by hydrolysis to glycerol and FFA, will be studied according to its component activities, i.e., tissue PHLA and hepatic PHLA.

Progress:

Part I.

Although the EPR studies provided no clear changes in membrane fluidity during the activation phase of CT interaction, certain properties of the membrane-bound AC under stimulation by CT and while treated with EPR spin labels were uncovered along with information relative to the position and environment of the AC enzyme.

Spin-labeled sterates (S-LS) have been used in EPR studies to determine the degree of motion in plasma membranes (PM). In this regard, different S-LS were used to measure the rigidity of thymocyte PM and to investigate possible interactions with the AC-cyclic AMP system. PM were found to be relatively rigid structures ($S=0.73$). Increasing concentrations (4-26 μM) of S-LS, but not their corresponding spin-labeled methyl sterates (S-LMS), increased membrane fluidity with 40% lysis and concomitant complete inhibition of CT-mediated increases in AC-cyclic AMP (206 ± 15 vs. 37 ± 3 pmol/ 10^7 cells, $p < 0.001$, compared to controls of 35 ± 4). Upon subsequent isolation of PM from these cells, CT-stimulated AC activity was likewise reduced (303 ± 26 vs. 63 ± 4 pmol/mg protein/min, $p < 0.001$, controls, 65 ± 5). S-LS but not S-LMS also inhibited isoproterenol, prostaglandin E_1 , and NaF-stimulated AC activity. Prior addition of S-LMS did not overcome

S-LS inhibition. Inhibition was complete within the first minute of addition to homogenates and required approximately 200 μ M S-LS for half-maximal inhibition. Unlabeled stearate and methyl stearate were not inhibitory. Inhibition occurred either in the presence or absence of an ATP regenerating system and was not readily reversible. In contrast, neither S-LS nor S-LMS inhibited membrane associated low Michaelis constant (K_m) AC-cyclic AMP phosphodiesterase activity. Action of S-LS was thought to be on the exterior of PM rather than interior. These results suggest the presence of multilipid domains and lipid-protein interactions in the AC-cyclic AMP system are consistent with the fluid mosaic model for PM. No further work is planned on this part.

Part II.

Studies on the effect of infection on the *in vitro* lipolytic activity of the fat depots was begun recently; results are inconclusive at this time. No further work will be done on this work unit.

Publications:

1. Petrella, V. J., and T. V. Zenser. 1976. Properties of cholera toxin-and NaF-stimulated adenylate cyclase from mouse thymocytes. *Biochim. Biophys. Acta* 421:237-245.
2. Zenser, T. V., V. J. Petrella, D. Seburn, and F. Hughes. 1976. Spin-labeled stearates as probes for the microenvironment of adenylate cyclase. *Clin. Res.* 24:373A.

LITERATURE CITED

1. Kimberg, D. V., M. Field, J. Johnson, A. Henderson, and E. Gershon. 1971. Stimulation of intestinal mucosal adenylate cyclase by cholera enterotoxin and prostaglandins. *J. Clin. Invest.* 50:1218-1230.
2. Beisel, W. R. 1972. Interrelated changes in host metabolism during generalized infectious illness. *Am. J. Clin. Nutr.* 25:1254-1260.
3. Kaufmann, R. L., C. F. Matson, A. H. Rowberg, and W. R. Beisel. 1976. Defective lipid disposal mechanisms during bacterial infection in rhesus monkeys. *Metabolism* 25:615-624.
4. Kaufmann, R. L., C. F. Matson, and W. R. Beisel. 1976. Hypertriglyceridemia produced by endotoxin: role of impaired triglyceride disposal mechanism. *J. Infect. Dis.* 133:548-555.
5. Neufeld, H. A., J. A. Pace, and F. E. White. 1976. The effect of bacterial infections on ketone concentrations in rat liver and blood and on free fatty acid concentrations in rat blood. *Metabolism* 25, in press.

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10. NO./CODES# a. PRIMARY 62760A	PROGRAM ELEMENT PROJECT NUMBER b. CONTRIBUTING	c. Classification CARDS 114(e)(f)		11. TASK AREA NUMBER 01	12. WORK UNIT NUMBER 005	
13. TITLE /Proceed with Security Classification Code# (U) Investigation of spontaneous diseases in laboratory animals				14. SCIENTIFIC AND TECHNOLOGICAL AREAS# 003500 Clinical medicine; 004900 Defense;		
15. START DATE 64 08	16. ESTIMATED COMPLETION DATE CONT		17. FUNDING AGENCY DA	18. PERFORMANCE METHOD C. In-house		
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23. RESPONSIBLE DOG ORGANIZATION NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701				24. PERFORMING ORGANIZATION NAME: Pathology Division USAMRIID ADDRESS: Fort Detrick, MD 21701		
25. RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833				26. PRINCIPAL INVESTIGATOR (Provide name if U.S. Government institution) NAME: Stookey, J. L. TELEPHONE: 301 663-7211 SOCIAL SECURITY ACCOUNT NUMBER:		
27. GENERAL USE Foreign intelligence considered				28. ASSOCIATE INVESTIGATORS NAME: Whitmire, R. E. NAME: Hickman, R. L. POC:DA		
29. KEYWORD (Proceed with Security Classification Code) (U) Laboratory animals; (U) Pathology; (U) Neoplasia; (U) Infectious diseases; (U) Parasites; (U) Military medicine; (U) BW defense (U) Streptococci						
30. TECHNICAL OBJECTIVE, 31. APPROACH, 32. PROGRESS (Provide individual paragraphs identified by number. Proceed with Security Classification Code.)						
23 (U) To define and quantitate the spontaneous diseases occurring in laboratory animals utilized at USAMRIID. This information is necessary to preclude or minimize the experimental variables of natural diseases, allow selection of adequate animal suppliers, control zoonosis and ultimately the successful completion of the laboratory BW research mission.						
24 (U) Investigate outbreaks of disease occurring in laboratory animals of the Institute.						
25 (U) 75 07 - 76 06 - During the past year 2,667 laboratory animal necropsies were performed to ascertain the level of disease in the Institute's experimental laboratory animals. These necropsies were performed as a result of and in support of individual research experiments, and in support of the quality control program for procured laboratory animals. Several of the instances of spontaneous disease in laboratory animals were selected for further research and study, the results of which have been or will be published. These include individual cases of neoplasia, parasitism, Sendai virus outbreaks in mice, and streptococcal meningoencephalitis with disseminated intravascular coagulation in a rhesus monkey.						
Publications: J. Am. Vet. Med. Assoc. 167:589, 648-650, 651-654, 1975. Vet. Pathol. 12:6-12, 1975. J. Parasitol. 62:111-115, 1976.						
Available to contractors upon ordinator's approval.						

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 005: Investigation of Spontaneous Diseases in Laboratory Animals

Background:

Normal colony laboratory animals are seldom completely free of disease. The vast majority of laboratory animals utilized by investigators in the Institute are procured from a variety of outside sources. It is imperative that a representative number of these animals be examined by veterinary pathologists in order to ascertain the quality of both the animals and their prior handling and environment. A variety of disease conditions can occur in laboratory animals, including infectious processes, parasitic infestations, nutritional imbalance and neoplasia. Some are unique to a species while others may affect a variety of animals, including man. Some diseases are present in these animals at the time they are procured and others may be manifested after their introduction into the Institute. In the former case it is important to identify diseased animals so that the contractor and the investigator can be informed. In the latter case, it is imperative that these disease conditions be detected and either eradicated or if this is not practical, brought to the attention of the investigators. An investigator is entitled to the best animals available but he should also be aware of the endemic disease processes inherent in the species of laboratory animals he selects for his experiment in order that he will not confuse them with induced experimental lesions.

Progress:

A total of 2,667 laboratory animal necropsies were performed this year in support of Animal Resources Division's quality control program and in support of a wide variety of research efforts of investigators throughout the Institute. These necropsies represent the entire spectrum of all laboratory animal species utilized in our research. The quality control program, to evaluate the health and ascertain the incidence of disease in all laboratory animals procured for use by investigators, has continued to be most useful and rewarding. This surveillance of newly purchased animals has pinpointed several conditions that may have compromised future research and has materially improved the quality of available research laboratory animals. The number of individual and group spontaneous disease outbreaks

occurring in our colony animals has been significantly decreased during the past year. Only a few relatively insignificant disease processes were observed and these will be individually summarized.

Several groups of guinea pigs were involved in high death losses during the year. In each case environmental stress factors, primarily excess heat and poor air circulation, were incriminated. However, in a recent disease outbreak, lesions of suppurative pneumonia, pleuritis, pericarditis and/or generalized peritonitis were encountered. Pure cultures of Streptococcus spp. were isolated. Environmental stress, namely high room temperatures, was also implicated in these death losses. Since guinea pigs are highly susceptible to both heat and streptococcal infection, this latter disease outbreak seems to combine the 2 factors.

In another interesting disease entity, a newly arrived, young, female rhesus monkey exhibited clinical CNS signs. Cultures of cerebrospinal fluid yielded a pure growth of Streptococcus pneumoniae; however, the monkey failed to respond to treatment and following a lengthy course of over 30 days was euthanized. In addition to severe necrotizing meningochoriomylitis, thrombi were noted in vessels of the spinal cord and glomerular capillaries. These findings strongly suggest that a spontaneous disseminated intravascular coagulation (DIC) was occurring in this monkey. DIC in both spontaneously and experimentally S. pneumoniae-infected rhesus monkeys has been observed in the past.

An iatrogenic outbreak of lymphosarcoma occurred in a group of mice in an experiment. Spleen cells from a donor mouse apparently contained malignant lymphocytes and their injection into recipient mice successfully transferred the inapparent malignancy to most of these mice, where the neoplasm grew rapidly and metastasized freely.

Two of our laboratory rabbits were found to harbor a parasite common in wild rabbits. Both rabbits contained multiple peritoneal cysts compatible with Cysticercus pisiformis, the intermediate stage of the cestode parasite Taenia pisiformis. The adult of this tapeworm is found in the intestine of the dog and other carnivores. The presence of these parasites in laboratory rabbits indicates an unsatisfactory source of supply where dogs are kept in close proximity to the rabbit hutches.

Occasional outbreaks of a respiratory disease in our young mice strongly suggest the presence of Sendai virus. Histopathology appears to confirm this diagnosis, with lung lesions compatible with those produced by this virus. Sendai virus is present as a contaminating, latent virus in many mouse colonies. It usually does not cause severe clinical disease but the morbidity is high among young animals.

Outside of our normal colony animals, an interesting and somewhat unusual lesion was encountered in an aged, male whitetail deer from a neighboring government reservation. This deer was semitame and frequently observed and fed. A rapidly growing mass was observed over one eye and it soon obscured the animal's vision on that side. A decision was made to destroy the deer and a necropsy was performed. The histologic diagnosis of the mass was fibrosarcoma. No metastases were found.

Presentations:

Stookey, J. L. Respiratory diseases of primates. Presented at course, "Pathology of Laboratory Animals," Armed Forces Institute of Pathology, Washington, D.C., 15-19 Sep 1975.

Publications:

1. Long, G. G., T. G. Terrell, and J. L. Stookey. 1975. Hepatomas in a group of captive woodchucks. *J. Am. Vet. Med. Ass.* 167:589.
2. Machotka, S. V., F. E. Chapple, III, and J. L. Stookey. 1975. Cerebral tuberculosis in a rhesus monkey. *J. Am. Vet. Med. Ass.* 167:648-650.
3. Long, G. G., J. D. White, and J. L. Stookey. 1975. Pneumocystis carinii infection in splenectomized owl monkeys. *J. Am. Vet. Med. Ass.* 167:651-654.
4. Moe, J. B., J. D. White, W. P. Czajkowski, and J. L. Stookey. 1975. Myxosarcoma in a young rhesus monkey. *Vet. Pathol.* 12:6-12.
5. Long, G. G., J. R. Lichtenfels, and J. L. Stookey. 1976. Anatrichosoma cynamolgi (Nematoda: Trichinellida) in rhesus monkeys. *J. Parasitol.* 62:111-115.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSED DA OE6410	2. DATE OF SUMMARY 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUMMARY 75 09 29	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SECY ^a U	6. WORK SECURITY U	7. REGRADING ^b NA	8. DA DISSEM INSTRN NL	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: ^c	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER 01	10. LEVEL OF SUM A. WORK UNIT 007	
a. PRIMARY	62760A	3A762760A824				
b. CONTRIBUTING						
c. CONFIDENTIAL	CARDS 114(e)(f)					
11. TITLE (Pecase with Security Classification Code) (U) Effect of infectious diseases on the coagulation mechanism						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^d 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry						
13. START DATE 72 08	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house			
17. CONTRACT/GRAANT	EXPIRATION:	18. RESOURCES ESTIMATE PROCUREMENTS	19. PROFESSIONAL MAN YRS 1.5	20. FUNDS (In thousands) 312		
a. DATES/EFFECTIVE:		FISCAL YEAR 76				
b. NUMBER: ^e	NA	CURRENT 77	1.0	103		
c. TYPE:						
d. KIND OF AWARD:						
21. RESPONSIBLE DOD ORGANIZATION	NAME: ^f USA Medical Research Institute of Infectious Diseases ADDRESS: ^f Fort Detrick, MD 21701	NAME: ^g Pathology Division USAMRIID ADDRESS: ^g Fort Detrick, MD 21701				
RESPONSIBLE INDIVIDUAL	NAME: Metzger, J. F. TELEPHONE: 301 663-2833	PRINCIPAL INVESTIGATOR (Punish 2040 if U.S. Academic Institution) NAME: Wing, D. A. TELEPHONE: 301 663-7351				
22. GENERAL USE	FOREIGN INTELLIGENCE Foreign intelligence considered	SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME: POC:DA				
23. KEYWORDS (Pecase each with Security Classification Code) (U) Infectious diseases; (U) Blood coagulation; (U) Monkeys; (U) Military medicine; (U) Salmonella typhimurium; BW defense						
24. TECHNICAL OBJECTIVE, ^h 25. APPROACH, 26. PROGRESS (Pecase individual paragraphs identified by number. Pecase each with Security Classification Code.)						
23 (U) Investigate the biochemical basis of blood coagulation dysfunctions arising as complications of infectious disease, especially those of potential BW significance. These complications, severe in their own right, often hinder effective treatment of the underlying disease process. Understanding the pathogenesis of coagulation dysfunctions will permit development of rational therapies to prevent or alleviate the condition.						
24 (U) Perform basic coagulation tests on samples from a variety of experimental infectious disease models. Correlate results with other indices of infection. Develop and test hypotheses concerning pathogenesis and biologic significance of observed changes. Evaluate effect of therapy.						
25 (U) 75 07 - 76 06 - An animal model of bacterial sepsis accompanied by a high incidence of disseminated intravascular coagulation has been established using S. typhimurium infection of rhesus monkeys (Macaca mulatta). The molecular mechanism triggering disseminated intravascular coagulation has been investigated comparing turnover of radiolabeled fibrinogen in infected versus control animals; an increased rate of fibrinogen catabolism was observed during episodes of disseminated intravascular coagulation resulting from infection.						
Two plasma proteins, alpha-2-macroglobulin and cold-insoluble globulin have been shown to be substrates for activated clotting factor XIII. Further, they both are apparently synthesized and secreted by fibroblasts.						
Publications: J. Biol. Chem 250:6614-6621, 1975, 251:1639-1645, 1976. J. Exp. Med. 142:462-467, 1976. Thromb. Res. 9:37-45, 1976.						
Available to contractors upon originator's approval.						

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 007: Effect of Infectious Diseases on Coagulation Mechanism

Background:

Appearance of an acquired hemorrhagic disorder introduces a serious complication to infectious disease which is not only severe in its own right, but may hinder treatment of the underlying disease. Associated with an increasing number of infectious diseases is the syndrome of disseminated intravascular coagulation (DIC). DIC is mediated by apparent simultaneous activation of the coagulation and fibrinolytic pathways, and leads to multiple coagulation defects characterized by IV fibrin formation, thrombocytopenia, consumption of clotting factors and increased fibrinolytic activity. An understanding of the molecular basis for the signs observed in DIC will aid in elucidating the pathophysiology of this syndrome, and thus lead to the development of rational therapeutic regimens for its control.

Equally important, activation of the coagulation mechanism is an integral part of the inflammatory response to infectious agents. Coagulation also serves to form the physical framework upon which the process of tissue repair is initiated. Investigation of the molecular basis for these events has been the subject of additional study.

A change in principal investigator for this work unit occurred this year, and this report reflects the contributions of both.

Progress:

Bacterial Sepsis. Studies were conducted to verify and document our observation that Salmonella typhimurium sepsis in monkeys was an infection model accompanied by DIC. That this is so, was confirmed for both Macaca mulatta and Macaca fascicularis. Inoculation IV of an appropriate dose of organisms into naive monkeys produces a week-long illness accompanied by a high incidence of DIC, but which is not necessarily fatal. Laboratory signs indicative of DIC, depressed fibrinogen levels, decreased platelet counts, and appearance of fibrin split products in the serum, could be correlated to clinical signs including the level of bacteremia, and extent of fibrile response.

Fibrinogen Turnover. Having established a model infection associated with DIC, this model was utilized in experiments designed to elucidate the mechanism by which an episode of DIC is triggered. Protein turnover studies were adopted as a tool to measure directly clotting factor utilization during infection.

M. mulatta fibrinogen was isolated by standard methods from pooled plasma and labeled with ^{14}C according to the method of Crane and Miller.¹ Catabolism of the ^{14}C fibrinogen was analyzed according to the model proposed by Atencio et al.,² and compared in infected and control rhesus monkeys. The catabolic half-life of labeled fibrinogen in control rhesus monkeys was observed to be 40-50 hr. Monkeys infected with 10^9 S. typhimurium had an illness accompanied by signs of DIC, and an increased rate of fibrinogen utilization. Monkeys infected with 10^8 organisms had an illness not accompanied by signs of DIC; their rate of fibrinogen catabolism was indistinguishable from that of control animals. Prompt antibiotic treatment did not significantly affect the clinical course of illness in monkeys given the higher dose of organisms, but did shorten the period of increased fibrinogen utilization.

Studies of α_2 Macroglobulin and Cold-insoluble Globulin. Cold-insoluble globulin (CIG) is a plasma protein possessing the electrophoretic mobility of a β -globulin, composed of 2 identical subunits each of 200,000 MW, and is often found in precipitates of so-called "cryofibrinogen," thought to be a marker for hypercoagulability and thus has been of some interest. Earlier work had established that CIG was substrate for fibrin stabilizing factor (FSF, clotting Factor XIII).³ In addition to fibrin and CIG, α_2 -macroglobulin (α_2 -macroglobulin ($\alpha_2\text{M}$) and an unidentified third protein were found to be plasma substrates for FSF on the basis of enzymic incorporation of the fluorescent amine dansylcadaverine into the proteins. Studies into the synthesis of CIG by fibroblasts⁴ led to the discovery that $\alpha_2\text{M}$ was also synthesized and excreted by these same cells.

Publications:

1. Mosher, D. F. 1975. Cross-linking of cold-insoluble globulin by fibrin-stabilizing factor. *J. Biol. Chem.* 250:6614-6621.
2. Mosher, D. F. 1976. Action of fibrin stabilizing factor on cold-insoluble globulin and α_2 -macroglobulin in clotting plasma. *J. Biol. Chem.* 251:1639-1645.
3. Mosher, D. F., and D. A. Wing. 1976. Synthesis and secretion of α_2 -macroglobulin by cultured human fibroblasts. *J. Exp. Med.* 143:462-467.
4. Mosher, D. F. 1976. Changes in plasma cold-insoluble globulin concentration during experimental Rocky Mountain spotted fever infection in rhesus monkeys. *Thromb. Res.* 9:37-45.

LITERATURE CITED

1. Crane, L. J., and D. L. Miller. 1975. A solid-phase radioimmunoassay for fibrinogen. *Anal. Biochem.* 64:60-67.
2. Atencio, A. C., H. R. Bailey, and E. B. Reeve. 1965. Studies on the metabolism and distribution of fibrinogen in young and older rabbits I. Methods and models. *J. Lab. Clin. Med.* 66:1-19.
3. Mosher, D. F. 1975. Cross-linking of cold-insoluble globulin by fibrin-stabilizing factor. *J. Biol. Chem.* 250:6614-6621.
4. Ruoslahti, E., and A. Vaheri. 1974. Novel human serum protein from fibroblast plasma membrane. *Nature* 248:789-791.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^b DA OA6414	2. DATE OF SUMMARY ^b 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUMRY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCY ^b U	6. WORK SECURITY ^b U	7. REGADING ^b NA	8. DSBIN INSTRN ^b NL	9. SPECIFIC DATA-CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: ^a PROGRAM ELEMENT				PROJECT NUMBER 3A762760A834	TASK AREA NUMBER 01	11. LEVEL OF SUM A. WORK UNIT 009
a. PRIMARY b. CONTRIBUTING c. FOREIGN	CARDS 114(e)(f)					
11. TITLE (Proceed with Security Classification Code) (U) Host amino acid, protein and RNA metabolism during infectious disease of military medical importance						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry						
13. START DATE 65 07	14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house		
17. CONTRACT/GRAANT						
a. DATES/EFFECTIVE:	EXPIRATION:			18. RESOURCES ESTIMATE	19. PROFESSIONAL MAN YRS	20. FUNDS (in thousands)
b. NUMBER: NA				FISCAL YEAR 76	2.0	265.6
c. TYPE:	d. AMOUNT:			CURRENT		
e. KIND OF AWARD:	f. CUM. AMT.			77	2.0	176.0
19. RESPONSIBLE DOD ORGANIZATION						
NAME: ^a USA Medical Research Institute of Infectious Diseases ADDRESS: ^a Fort Detrick, MD 21701						
20. PERFORMING ORGANIZATION						
NAME: ^a Physical Sciences Division USAMRIID ADDRESS: ^a Fort Detrick, MD 21701						
PRINCIPAL INVESTIGATOR (PUNNED DEAN II U.S. Academic Institution) NAME: ^a Wannemacher, Jr., R. W. TELEPHONE: 301 663-7181 SOCIAL SECURITY ACCOUNT NUMBER:						
ASSOCIATE INVESTIGATORS NAME: Thompson, W. L. NAME: Beisel, W. R.						
POC:DA						
21. KEYWORDS (Proceed EACH with Security Classification Code) (U) Amino Acids; (U) Alanine; (U) Gluconeogenesis; (U) Catabolism; (U) Volunteers; (U) Skeletal muscle; (U) RNA synthesis; (U) Protein synthesis; BW defense						
22. TECHNICAL OBJECTIVE, 23. APPROACH, 24. PROGRESS (Furnish individual paragraphs identified by number. Proceed with Security Classification Code.)						
23 (U) Changes in the concentrations and pattern of amino acids in blood and tissues during various infectious diseases will be correlated with alterations in RNA, protein and carbohydrate metabolism. The data obtained will be utilized to develop nutrient therapy to prevent the body wasting which is associated with infectious disease. This in turn, should shorten the time of convalescence for these illnesses. In addition, alterations in the concentration or ratio of some blood amino acids may be a useful biochemical tool in the early detection of infectious diseases that pose a potential BW threat to this country						
24 (U) Free amino acid concentrations are determined by ion-exchange chromatography in plasma and tissue of experimental subjects infected with bacterial or viral organisms. Radioactive nonmetabolizable and metabolizable amino acid tracers are utilized to study amino acid flux. Labeled precursors of RNA and protein metabolism are used to study effects of infection on RNA and protein synthesis.						
25 (U) 75 07 - 76 06 - Total body protein catabolism is increased in infected rats above that associated with simple starvation. This is coupled with increased gluconeogenic capacity of liver, elevated rate of release of alanine from muscle tissue and increased glucose oxidation and turnover. The inability of the infected host to develop starvation ketosis may be related to increased catabolic rates. Infection increased synthesis of both hepatic ribosomal RNA and messenger RNA associated with the bound ribosomal fraction.						
Publications: Biochem. J. 156:25-32, 1976. J. Lab. Clin. Med. 87:577-585, 1976. Fed. Proc. 35:343, 1563, 1976. Am. J. Clin. Nutr. 29:997-1006, 1976						
Available to contractors upon originator's approval.						

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 009: Host Amino Acid, Protein and RNA Metabolism During Infectious Disease of Military Medical Importance

Background:

Part I.

It has been postulated that the increased catabolism which is associated with infectious disease is the result of utilization of body proteins to supply amino acids for gluconeogenic and protein anabolic processes associated with host defense (such as synthesis of proteins associated with humoral and cell-mediated immunity, acute-phase globulins, etc.).¹ It has also been demonstrated that skeletal muscle is the major contributor of the amino acids which are utilized by the host defenses during infectious disease.² This results in an alteration of the plasma free amino acids, markedly different from that observed during starvation.³ Because of these differences studies were initiated to determine the effect of infectious process on the release and utilization of individual amino acids. In addition, a model has been developed to measure the effects of infection and/or starvation on overall rates of body catabolism.

Part II.

It has been previously shown that in response to infection, LEM treatment or inflammatory processes (such as turpentine abscess), there is an increase in the uptake of amino acids into hepatic cells and a concomitant rise in the serum concentration of acute-phase proteins.⁴ Studies were initiated to determine the role of RNA in this process. These studies involve the subfractionation of hepatic cells from control and treated rats and the isolation, quantitation and characterization of the RNA species from these various subfractions.

Progress:

Part I.

An experimental model was sought to measure the effects of infection on the rate of catabolism of body proteins. When a rat is injected with a dose of labeled amino acids, the radioactivity is rapidly lost from the

free amino acid pools and those proteins with rapid turnover rates, such as found in liver and the visceral tissues. By 14 days after injection of labeled amino acid most of the radioactivity is associated with the skeletal muscle and skin of the rat. Phenylalanine is a unique amino acid in that it can only be metabolized by liver and perhaps kidney, which contain the rate-limiting enzyme, phenylalanine hydroxylase. Therefore, rats were force-fed ^{14}C -phenylalanine and maintained on a basal diet containing 18% casein for 14 days. By this time free amino acid pools contained virtually no labeled amino acids while the proteins of skin and skeletal muscle contained \sim 75-80% of the label. The rats were then placed in a metabolic cage which allowed for complete 24-hr collection of expired CO_2 , urine and feces. In one study, food was withheld from the rats when they were placed in the metabolic cage and during the subsequent collection periods, while in the second study rats had access to food from 1600-0800 hours. After 24 hr in the metabolic cages, rats were injected with 10^4 *Streptococcus pneumoniae* or equivalent numbers of heat-killed organisms. Complete CO_2 , urine and fecal collections were then made for the next 2 days. The rats were then killed and the body separated into skin, carcass, liver and viscera for subsequent hydrolysis and the determination of proteins and ^{14}C content. During the first day the rats lost \sim 4% of the radioactivity in expired CO_2 , urine and feces, with the CO_2 accounting for almost 90% of this loss. In fed rats, diurnal periodicity was observed with lower rates of catabolism during the daytime hours and higher rates during the dark nighttime. During the first day of starvation, significantly greater amounts of protein catabolism were observed during the night. Subsequent starvation resulted in a greater rate of protein catabolism during the night hours and eventual loss of diurnal periodicity. This resulted in a significantly greater rate of protein catabolism during the third day of starvation as compared to fed rats. A *S. pneumoniae* infection imposed upon starvation resulted in even greater rates of protein catabolism during the dark hours and a complete loss of periodicity during the second day. A significant increase in body protein catabolism was observed during the first or second 24 hr after exposure to *S. pneumoniae* when compared to either the fed or starved controls. The increase in protein catabolism correlated with elevated negative nitrogen balance and excretion of 3-methylhistidine and hydroxyproline. Following 72 hr of starvation, the percentage of proteins or radioactivity was significantly less in the liver as compared to fed controls. However, the liver, viscera, skin or carcass of the infected rat had a similar distribution of protein and radioactivity to that found in the starved rats. Thus, the additive effects of infection resulted in increased catabolism of total body proteins while early starvation resulted in increased catabolism of labile proteins especially those associated with liver.

Severe sepsis in man results in a doubling of the rate of glucose turnover and oxidation. To meet the increased glucose needs the body would

have to elevate its rates of amino acid gluconeogenesis. Alanine is considered to be the major amino acid which is utilized by the liver for glucose production. Studies in the perfused liver suggested that the infected rat was not able to utilize Ala as effectively for gluconeogenesis as was that of heat-treated controls. Therefore, studies were initiated in the rat infected with S. pneumoniae to determine the in vivo rates of gluconeogenesis and glucose utilization. In general, these studies utilized a pulse-dose of labeled Ala to assess gluconeogenesis; in in vitro studies an excess of Ala, usually 10 mM, was added to the perfusate. Therefore, in vivo models were developed to evaluate the effects of infection on glucose production from either a pulse-dose or load of Ala. In each study rats were injected SC with either 10^4 or 10^8 heat-killed or virulent S. pneumoniae. All measurements of gluconeogenic rate were performed 24 hr after the injection. At this time, the rats injected with the lower dose of virulent organisms were febrile, septic, and demonstrated acute signs of clinical illness. In contrast, the rats receiving the higher dose were hyperthermic, severely ill, rarely survived beyond 30 hr, and were in the agonal stages of the infection. When given a pulse-dose of labeled Ala both groups of infected rats contained more labeled glucose than did their fasted controls. When rats were given the Ala load the acutely ill group contained significantly more labeled glucose than did their pair-fed controls. However, total body glucose or ^{14}C -glucose was significantly lower during the agonal stages of the infection. In liver perfusion or isolated hepatocyte studies, livers from rats during the acute stage of the infection produced similar or increased amounts of glucose from Ala or pyruvate compared to the noninfected controls. Again, glucose production was significantly decreased during the agonal stages of the infection. From these in vivo and in vitro studies it is suggested that during the acute-phase of pneumococcal infection in the rat the gluconeogenic potential of the liver is increased. However, during the agonal stages of infection rats were unable to handle these gluconeogenic substrates as efficiently as their pair-fed controls. By use of a pulse-dose of ^{14}C -U-glucose it is possible to demonstrate that during both the acute and agonal stages of the pneumococcal infection, glucose turnover and oxidation were significantly increased above noninfected controls. Since the infection-related anorexia will result in a rapid depletion of the glycogen stores, the host must depend on amino acids as substrates for glucose production. The reduced gluconeogenic capacity of the liver in the agonal stages of the infectious process may explain the hypoglycemia which is observed in this stage of some infections in man.

Since it has been proposed that infections in man and experimental animals stimulate a flux of amino acids from skeletal muscle, a technique was sought to confirm this. Amino acid release has been measured by arterial venous differences across skeletal muscle vasculature, by hind quarters perfusion technique, or by in vitro release of amino acids by an intact muscle from the forearm of rats. In the latter technique, the dorsal epitrochlearis branchii muscle of the forearm was carefully dissected, placed in

Krebs-Ringer bicarbonate (pH 7.4) and incubated at 37 C for 30 min. After incubation the supernatant was analyzed for free amino acid concentrations. The total amino acids released were 2 to 3 times more than were present in the protein-free filtrate of similar muscles before addition of the incubation medium. Glutamine and Ala constitute 45 to 50% of the total amino acid release from skeletal muscle. The pattern of release by the in vitro technique was very similar to that reported for the perfused-hind quarter of the rat or by arterial-venous differences from the forearm of man. The higher concentrations of Glu and Ala have been interpreted by others to suggest that these 2 amino acids are utilized to carry nitrogen to skeletal muscle to liver for use in ureagenesis. In addition, Ala has been shown to be the major gluconeogenic amino acid; it has been suggested that pyruvate from glycolysis and the carbon skeleton of ketoacids in skeletal muscle can be converted to Ala which is then transported to the liver and utilized for glucose synthesis. Twenty-four hr after exposure to S. pneumoniae the skeletal muscle released more Glu and Ala in the in vitro system than did muscles from noninfected controls. Thus, this infection in rats induces greater transamination of amino acids in skeletal muscle, especially those associated with the branched-chain amino acids. The ketoacids would then be utilized by the skeletal muscle as a source of energy or conversion to pyruvate and Ala to serve as substrates for elevated rates of gluconeogenesis. The branched-chain amino acids (Val, Leu, and Ile) are catabolized mainly in the skeletal muscle and may serve as a source for ketone production in this tissue. In muscle of the infected rats less branched-chain amino acids were released than in noninfected controls. The release of Phe was increased while that of Tyr was decreased in the skeletal muscles of infected rats. This resulted in elevation in the Phe:Tyr ratio, which may be the origin of the elevated ratios which are routinely observed in the serum of man and experimental animals with various types of inflammatory diseases.

From these experiments, mechanisms are proposed to explain some of the infection-related alterations in host protein metabolism. Decreased dietary intake results in a depression of circulating glucose. To meet the energy needs of some of the peripheral tissue the body stimulates lipolysis of the adipose stores and the development of a starvation ketosis. With decreases in glucose supply, skeletal muscle protein breaks down and certain amino acids (mainly branched-chain) are utilized for keton production. The NH₃ produced during this amino acid catabolism is coupled with pyruvate which results in increased release of Ala to be taken up by the liver as a substrate for gluconeogenesis. As starvation continues the body increases its hepatic ketone production and turns off the utilization of amino acids by skeletal muscle as an energy source. Recently, it has been shown that infection prevents the starvation-induced ketosis (Work Unit 834 01 022). Thus, the skeletal muscle must continue to utilize its amino acids as a source of energy. The NH₃ produced during this catabolism is coupled to Ala or Glu,

which is released in increased quantities by the skeletal muscle, and is utilized by the liver and kidney respectively for gluconeogenesis, ureagenesis and ammoniagenesis. The carbon skeleton of amino acids, such as the branched-chain, can be used as a source of energy, while others can be converted to pyruvate and α -ketoglutarate for transamination to Ala and Glu. This results in a decrease in plasma concentration in these amino acids. Certain amino acids such as Phe, Tyr, and Try are not metabolized by skeletal muscle. The infection-related increase in skeletal muscle catabolism results in elevated release of these amino acids from muscle and subsequent rise in the plasma concentration. Therefore, the inability of the infected host to develop starvation ketosis results in the continual utilization of amino acids as a source of energy and a marked elevation in the rate of wasting of body protein as compared to simple starvation.

Part II.

It has been previously reported that there is an increase in the production of RNA, particularly in the bound ribosomal fraction in response to infection, LEM treatment, or turpentine abscess.⁵ However, when these same experiments were run on adrenalectomized (Adrex) and hypophysectomized (Hypox) rats, no significant change in RNA production was observed. Serum levels of α_2 -macroglobulin (α_2 -MFP), which increase in a normal animal, showed no response in the Adrex and Hypox rats, while the level of haptoglobin was increased under both conditions. When Adrex and Hypox rats were maintained on daily injections of hydrocortisone, increases in RNA production and serum α_2 -MFP were once again observed.

Subsequent experiments have shown that injection of actinomycin D into LEM-treated rats inhibits the normal increase in α_2 -MFP but not haptoglobin. While an injection of cycloheximide completely inhibits LEM-stimulation of both these proteins. Thus, it appears that, (1) glucocorticoids are necessary for eliciting increased transcriptional rates of RNA in response to stress conditions; and (2) the production of certain acute-phase proteins, such as haptoglobin, may not require the synthesis of new mRNA, but the utilization of previously existing mRNA, a process which does not require the presence of glucocorticoids.

The isolation of free and bound mRNA using phenol-chloroform for extraction of RNA, Oligo-dT-cellulose for separation of the mRNA from rRNA, and dialysis and lyophilization for concentration, have shown that there is a larger percentage of mRNA associated with the free ribosomes (~ 0.3% of total RNA) as compared to the bound (~ 0.05% of total RNA). However an increase in the amount of bound mRNA produced has been observed in response to infection and turpentine treatment.

Testing of this mRNA by Dr. Abeles in the in vitro wheat germ embryo system has shown an ability for the free mRNA to translate, but little if any activity for the bound mRNA. Since ribosomal RNA patterns of the material which was lyophilized were not typical, new methods are currently being employed for isolation, extraction and concentration of free and bound ribosomes. Bound ribosomes are isolated using only a mild detergent to strip off the nuclei. Both fractions are extracted with detergent rather than phenol-chloroform and layered directly on Oligo-dT cellulose columns. The mRNA is then precipitated directly with ethanol using tRNA as a carrier if needed. Using these techniques, 100% of the RNA is recovered in the various fractions. Electron micrographs of free and bound ribosomes have provided good physical evidence of separation of these 2 fractions. Characterization on sucrose density gradients and in vitro translational activity have not been fully assessed.

In vitro studies on isolated free and bound ribosomes have been conducted to determine their ability to incorporate amino acids. While the bound ribosomes show a reduced rate of incorporation when compared to the free ribosomes on the basis of total RNA, preliminary data suggest that both fractions are comparable in incorporation rate when expressed in terms of the amount of mRNA added to the system. In vitro assays on free and bound ribosomes from control and infected rat livers have shown that there is no increase in translational rate when expressed in terms of either total RNA or mRNA. This indicates that the increase in production of serum proteins in response to infection is due to an increase in the production of ribosomal and mRNA and not an improvement in translational activity.

Presentations:

1. Wannemacher, Jr., R. W., and W. R. Beisel. Effect of infection on host metabolism. Presented, at the Xth International Congress of Nutrition Symposium: Nutrition and Infection, Kyoto, Japan, 2-9 August 1975 (Abstracts of papers, p. 75).

2. Wannemacher, Jr., R. W., H. A. Neufeld, and P. G. Canonico. Hepatic gluconeogenic capacity and rate during pneumococcal infection in rats. Presented, Annual Meeting FASEB, Anaheim, CA, 11-16 April 1976. (Fed. Proc. 35:343, 1976).

3. Wannemacher, Jr., R. W., A. S. Klainer, G. A. Higbee, and W. R. Beisel. Trace element changes during infection at various ages. Presented, Conference on the "Biomedical Role of Trace Elements in Aging", Eckerd College, St. Petersburg, FL, 28-30 April 1976.

4. Wannemacher, Jr., R. W. Key role of various individual amino acids in host response to infection. Presented, Workshop on Impact of Infection on Nutritional Status of the Host, National Research Council, Airlie House, VA, 11-13 May 1976.

5. Wannemacher, Jr., R. W., and R. E. Dinterman. Total body protein catabolism in starved and infected rats. Presented, Annual Meeting, American Society of Biological Chemists, San Francisco, CA, 6-10 June 1976. (Fed. Proc. 35:1563, 1976).

Publications:

1. Thompson, W. L., F. B. Abeles, F. A. Beall, R. E. Dinterman, and R. W. Wannemacher, Jr. 1976. Influence of the adrenal corticoids on the stimulation of synthesis of hepatic ribonucleic acid and plasma acute-phase globulins by leukocytic endogenous mediator. Biochem. J. 156:25-32.

2. Bostian, K. A., B. S. Blackburn, R. W. Wannemacher, Jr., V. G. McGann, W. R. Beisel, and H. L. DuPont. 1976. Sequential changes in the concentration of specific serum proteins during typhoid fever infection in man. J. Lab. Clin. Med. 87:577-585.

3. Wannemacher, Jr., R. W., A. S. Klainer, R. E. Dinterman, and W. R. Beisel. 1976. The significance and mechanism of an increased serum phenylalanine-tyrosine ratio during infection. Am. J. Clin. Nutr. 29: in press.

4. Wannemacher, Jr., R. W., and R. E. Dinterman. 1976. Total body protein catabolism in starved and infected rats. Workshop on Impact of Infection on Nutritional Status of the Host. Am. J. Clin. Nutr., in press.

LITERATURE CITED

1. Wannemacher, Jr., R. W. 1975. Protein Metabolism, p. 85-153. In Total Parenteral Nutrition: Premises and Promises. (ed. H. Ghadimi), John Wiley and Sons, Inc., New York.

2. Wannemacher, Jr., R. W., R. E. Dinterman, R. S. Pekarek, P. J. Bartelloni, and W. R. Beisel. 1975. Urinary amino acid excretion during experimentally induced sandfly fever in man. Am. J. Clin. Nutr. 28:110-118.

3. Wannemacher, Jr., R. W., R. S. Pekarek, P. J. Bartelloni, R. T. Vollmer, and W. R. Beisel. 1972. Changes in individual plasma amino acids following experimentally induced sand fly fever virus infection. Metabolism 21:67-76.

4. Wannemacher, Jr., R. W., R. S. Pekarek, W. L. Thompson, R. T. Curnow, F. A. Beall, T. V. Zenser, F. R. DeRubertis, and W. R. Beisel. 1975. A protein from polymorphonuclear leukocytes (LEM) which affects the rate of hepatic amino acid transport and synthesis of acute-phase globulins. Endocrinology 96:651-661.

5. U. S. Army Medical Research Institute of Infectious Diseases. 1 July. 1975. Annual Progress Report, FY1975, p. 15-22, Fort Detrick, Frederick, MD.

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20. RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833	POC:DA					
21. GENERAL USE Foreign intelligence considered	22. TECHNICAL OBJECTIVE, 23. APPROACH, 24. PROGRESS (Punish individual paragraphs identified by number. Proceed rest of each with Security Classification Code.) (U) Radiation; (U) Mice; (U) Infectious diseases; (U) Vaccine; (U) Military medicine; (U) Encephalomyelitis, equine (VEE); BW defense (U) Radiation exposure from nuclear attack will have a detrimental effect on immune and physiologic processes of military and civilian personnel possibly exposed to a simultaneous BW attack. This work unit is aimed at investigating interrelationships between acute or chronic irradiation and immune or disease processes in animal models, so that limitations of protective vaccines or disease in irradiated personnel may be more effectively managed in a nuclear warfare environment. (U) Acute or protracted whole body irradiation is delivered to selected animal species before, simultaneously with, or after infection. Clinical and immune responses are observed and measured serially. (U) 75 07 - 76 06 - Ultimate percentage survival in mice inoculated with virulent VEE virus can not be changed significantly by prior total-body irradiation, but the median time to death is shortened at higher radiation exposure doses. Brain virus levels are lower in irradiated, infected mice than in nonirradiated, infected animals. Brain lesions, which are progressively more severe with increasing virus dose, can be suppressed by total-body irradiation exposures of 200-600 R, which indicates that an immune component probably contributes to lethality from this highly virulent infection. As has been reported for endotoxins, total-body irradiation prolongs survival and increases percentage survival in animals inoculated with lethal doses of staphylococcal enterotoxin B. Neither a preexisting bacterial or viral infection is able to alter vascular kinetics of TC-83 virus in irradiated or normal monkeys. Both the total humoral antibody response and antibody subclasses IgM and IgG are delayed in their appearance in the serum of irradiated monkeys inoculated with TC-83 virus, but the typical IgM-IgG pattern is otherwise unaltered.					
Publications: Infect. Immunity 12:592-601, 1975. Lab. Anim. Sci. 26:436-442, 1976.						

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BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 010: Effect of Ionizing Radiation on Immunity and Pathophysiology of Infectious Diseases

Background:

Immunosuppression, whether accomplished by irradiation, drug, or chemical treatment, has been shown to affect the recovery of animals from experimental viral infections.¹ Most of the evidence points to increased susceptibility when these hosts are subjected to immunosuppressive treatments.^{1, 2} In other models where the host was highly susceptible to a particular virus, further enhancement of infection has been difficult to achieve.¹ Although virulence, morbidity, and mortality following experimentally induced arbovirus encephalitis have been enhanced in cyclophosphamide-treated rodents, survival time was often increased.¹ In some experimental neurotropic viral infections in rodents, less severe brain pathology, as well as prolonged survival time, was noted when the cell-mediated immune response was suppressed.³ These results suggest that the pathogenesis of certain viral encephalitides may be modified by alterations of the inflammatory response. In view of earlier studies at this laboratory on VEE virus, it was of interest to determine if the inflammatory response observed in the brains of infected mice were alterable by prior treatment with total-body irradiation.

There are several reports in the literature of radiation-induced protection and prolonged survival in animals challenged with endotoxins. In view of this, studies are in progress to assess the relative role of total-body irradiation in protecting rabbits from lethal IM doses of SEB and in prolonging the survival of irradiated monkeys given SEB.

Previous studies in monkeys given the VEE virus vaccine strain, TC-83, have shown that viremia is delayed and prolonged, and serum virus titers are elevated in irradiated, TC-83-vaccinated monkeys. It was hypothesized that the viremic changes may be in part related to changes in virus clearance kinetics in infected monkeys; the kinetics of radiolabeled TC-83 have been studied in normal, irradiated, infected (17-D yellow fever and Salmonella typhimurium) and irradiated, infected monkeys.

Progress:

Immunosuppression to assess the role of immune response in VEE-infected mice

Preliminary studies indicated that morbidity may be prolonged in Trinidad strain VEE (VEE-1) virus-infected mice following immunosuppressive treatment.

We have semiquantitatively graded the severity of encephalitis in irradiated and nonirradiated, infected mice and attempted to relate these changes with survival.

The effect of VEE-1 virus on the laboratory mouse, particularly in destruction of myeloid and lymphoid elements, followed by total paralysis, has been well described⁴ and is consistent with our observations. Histologic examination of the brains revealed significant differences between sick mice of both nonirradiated and irradiated groups at several virus dilutions. The sequential development of brain lesions following infection from several histopathologic studies included vasculitis, gliosis, neuronal necrosis and, finally, total necrosis with destruction of identifiable anatomic features. The temporal pattern of brain lesion development from one such study is shown in Table I.

It was interesting to find that prior x-irradiation exposure of mice to 600 R virtually eliminated lesions of vasculitis and encephalitis (Table I). In irradiated, infected mice, neuronal necrosis was not consistently observed in 100% of the animals in a group on any one particular day of sacrifice, as occurred on several of the days in the nonirradiated, infected groups (Table I). However, the severity of neuronal lesions, when observed, was not different in the 2 groups.

It is clear that prior irradiation exposure of mice did not potentiate encephalitis or lethality when mice were inoculated by the IP route. Similarly, brain virus levels of irradiated mice were not greater, and in some cases were lower, than those of nonirradiated, infected controls.

As a follow-up study, we examined survival of VEE-virus-infected and irradiated, infected mice based on scoring live/dead from large populations of mice for 30 days. Irradiation was accomplished 4 days before infection with radiation exposure doses of 200, 400, or 600 R. VEE-1 virus inocula were 10^1 , 10^2 , 10^3 , or 10^4 MIPLD₅₀. Percentage survival at 20 or 30 days was not significantly enhanced by irradiation except in the higher virus-dose groups (10^2 and 10^3 MIPLD₅₀), where a slight increase in survival was obtained by prior radiation exposure to 200 R. Another population of mice of the same strain was used to evaluate histopathology in 6 areas of the brain (Table II), and to score brain-lesion severity in each area. Mice from both groups were killed on days 3, 5, 7, 10, 14, or 21 postinfection. Severity was scored according to a previously described method⁵ by one pathologist without any knowledge as to the identity of experimental animals by group. Brain lesion scores were averaged for each group by day, and the highest mean scores for a particular day are shown in Table II.

TABLE I. HISTOPATHOLOGIC LESIONS IN INDIVIDUAL IRRADIATED AND NONIRRADIATED MICE INFECTED WITH VEE-1 VIRUS

DAY POST- INFECTION	VAS CULITIS					ENCEPHALITIS AND/OR		
	100 MIPLD ₅₀		10 MIPLD ₅₀			100 MIPLD ₅₀		
	Only XRT ^a	Only VEE-1	XRT + VEE-1	Only VEE-1	XRT + VEE-1	Only XRT	Only VEE-1	XRT + VEE-1
3	0 ^b	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
4	0	2	0	0	0	0	0	0
	0	1	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
	0	3	0	0	1	0	0	0
	0	1-3	0	0	0	0	0	0
	0	0	0	0	2	0	0	0
5	0	3	0	0	0	0	1	0
	0	3	0	0	0	0	0	0
	0	3	0	0	0	0	2	0
	0	4	0	2	0	0	2	0
	0	0	0	3	0	0	0	0
	0	1-2	0	0	0	0	1	0
6	0	4	0	3	0	0	2-3	0
	0	3-4	0	3	2	0	1-2	0
	0	4	1	4	0	0	2-3	0
	0	3-4	0	4	0	0	3-4	0
	0	4	0	0	2	0	3	0
	0	4	0	0	0	0	3	0
7	0	4	0	3	1	0	4	0
	0	3-4	0	3	0	0	3	0
	0	4	0	0	0	0	4	0
	0	4	0	4	0	0	3	0
	0	3-4	0	0	0	0	3	0
	0	2-3	0	0	0	0	2	0
8	0	3	0	2	0	0	3	0
	0	4	0	4	0	0	3	0
	0	4	0	2-3	0	0	3	0
	0	4	0	0	0	0	3	0
	0	3	0	0	0	0	3	0
	0	3	0	0	0	0	3	0

^aXRT = x-radiation treatment (600 R).

^b0 = no significant lesions.

^c1 = minimal.

^d2 = mild.

^e3 = moderate.

^f4 = severe.

TABLE I, continued

MENINGOENCEPHALITIS		NEURONAL NECROSIS				
10 MIPLD ₅₀		100 MIPLD ₅₀			10 MIPLD ₅₀	
Only VEE-1	XRT + VEE-1	Only XRT	Only VEE-1	XRT + VEE-1	Only VEE-1	XRT + VEE-1
0	0	0	0	0	0	1 ^e
0	0	0	0	0	0	0
0	0	0	0	0	0	1
0	0	0	0	0	0	0
0	0	0	0	0	1-2 ^d	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	1	0	0	3 ^e
0	0	0	1	3	2	2
0	0	0	0	0	2	0
0	0	0	3-4 ^f	1-4	0	1
0	0	0	1-3	0	0	0
0	0	0	0	0	2	2-3
0	0	0	2	2-4	3	0
0	0	0	3	2	0	0
0	0	0	3	0	0	0
0	0	0	2-3	0	2	0
2	0	0	0	4	2-4	0
0	0	0	2-3	1-2	3	0
2	0	0	3-4	0	2	0
2	0	0	3-4	0	3-4	1-4
3	0	0	2-3	4	3-4	0
3	0	0	3-4	3-4	3-4	3
3	0	0	3-4	3	0	3
0	0	0	2-4	0	0	2-3
0	0	0	2-3	3-4	2-3	1-2
2-3	0	0	3	3-4	2	1
3	0	0	2-3	0	0	0
0	0	0	3-4	0	3-4	0
3	0	0	3-4	0	0	0
0	0	0	3-4	0	0	0
0	0	0	2-3	0	0	2-3
4	0	0	4	0	3	0
4	3-4	0	4	0	3	0
4	0	0	4	0	4	0
0	0	0	4	0	0	0
0	0	0	4	0	0	0
0	0	0	4	0	0	0

TABLE II. MEAN PEAK BRAIN LESION (MPBL) AND SCORE/DAY POSTINFECTION

MIPLD ₅₀ AND SITE	MPBL/DAY BY RADIATION DOSE (R)			
	0	200	400	600
<u>Olfactory bulb</u>				
10 ¹	0.67/5	1.83/7	0.83/5	0.33/5, 7
10 ²	2.17/5	2.83/7	2.5/7	2.17/5
10 ³	3.3/7	2.4/5	2.6/7	2.67/5
10 ⁴	3.67/7	3.17/7	2.83/7	2.5/5
Radiation control	--	0.3/14	0.17/7	0.3/14
Room control	0.0	--	--	--
<u>Hippocampus</u>				
10 ¹	0.3/5, 7	0.67/7	0.3/5	0.2/5
10 ²	1.3/5	1.67/5	1.83/7	0.83/5
10 ³	2.3/7	1.67/5, 7	2.0/7	1.0/5
10 ⁴	2.17/7	1.83/5, 7	2.0/7	1.0/5
Radiation control	--	0.17/14	0.17/5, 7	0.3/14
Room control	0.3/14	--	--	--
<u>Thalamus</u>				
10 ¹	0.67/5	0.83/7	0.67/5	0.17/5
10 ²	1.83/5	2.5/7	2.0/7	1.17/5
10 ³	3.0/7	1.83/5	2.0/7	1.83/5
10 ⁴	3.0/7	2.67/7	2.0/7	1.5/5
Radiation control	--	0.5/10	0.5/5	0.5/14
Room control	0.5/5	--	--	--

TABLE II, continued

MIPLD ₅₀ AND SITE	MPBL/DAY BY RADIATION DOSE (R)			
	0	200	400	600
<u>Lenticulostriate</u>				
10 ¹	0.3/7	0.83/7	0.3/5	0.3/21
10 ²	1.83/5	2.17/7	2.0/7	1.17/5
10 ³	2.67/7	2.0/5	2.0/7	1.83/5
10 ⁴	3.0/7	2.5/7	2.0/7	1.83/5
Radiation control	--	0.3/5, 14	0.17/7	0.3/14
Room control	0.5/5	--	--	--
<u>Cerebral cortex</u>				
10 ¹	0.3/7	0.83/7	0.3/5	0.3/5
10 ²	1.3/5	1.67/5	2.0/7	0.3/5, 21
10 ³	2.0/7	1.3/7	2.0/7	1.17/5
10 ⁴	2.3/7	2.17/7	1.67/5	0.8/7
Radiation control	--	0.0	0.0	0.17/14
Room control	0.0	--	--	--
<u>Medulla oblongata</u>				
10 ¹	0.5/5	0.83/7	0.3/5	0.17/5
10 ²	1.5/5	1.5/5	1.3/7	0.3/5, 21
10 ³	2.0/5, 7	1.5/5	1.83/7	0.83/5
10 ⁴	2.5/7	2.17/7	1.3/7	0.3/5
Radiation control	--	0.0	0.0	0.17/14
Room control	0.0	--	--	--

In all 6 areas of the brain examined (olfactory bulb, thalamus, cerebral cortex, hippocampus, lenticulostriate, and medulla oblongata) MPBL scores were directly proportional to the dose of virus. Mean scores for mice in radiation control groups were not different from those found for the room controls (no virus, no irradiation). MPBL scores increased slightly in the 200 and 400 R groups when the virus inoculum was low ($\leq 10^2$ MIPLD₅₀). To the contrary, for mice in the high groups, $\geq 10^3$ MIPLD₅₀, they were inversely related to radiation exposure dose.

It is clear from the histopathology data in this series of experiments that prior total-body irradiation exposure suppressed much of the devastating destruction expected in vulnerable brain areas of nonirradiated, inoculated mice. Much of this destruction can be accorded to inflammatory cells and their associated debris, which is clearly less prominent in previously irradiated animals. Radiation immunosuppression clearly does not enhance this arbovirus infection of relatively high IP virulence. Even though x-radiation exposure suppressed MPBL scores, ultimate survival from VEE-1 virus infection was not altered significantly in the irradiated host. Median survival time was not enhanced in radiation-suppressed mice inoculated with this virus, which is contrary to reports with other arbovirus infections.

These studies indicate that the inflammatory response contributed to total morbidity and mortality from this acute infection. Radiation exposure suppressed inflammation, or otherwise induced anti-inflammatory effects which reduced the severity of pathologic findings early (5-7 days postinoculation), but did not greatly enhance ultimate survival of the host. This is consistent with the findings of others⁶ in suppressed mice treated with 17-D yellow fever and herpes hominus infections.

Radiation studies with SEB.

Collaborative studies in physiology (Work Unit No. 834 01 110) were conducted to evaluate the effects of IV SEB (0.05 mg/kg) on selected cardiorenal functions of rhesus monkeys exposed to 400 R of x-radiation, the latter given 4 days before SEB injection. During 5 hr after injection, cardiac output, glomerular filtration rate, and renal blood flow in x-irradiated monkeys were not as depressed as occurred with similar measurements in nonirradiated monkeys given SEB. Irradiated, SEB-treated monkeys survived longer, ~ 90 hr vs. 15-20 hr, than SEB-treated control animals. To pursue the prolonged survival observation, we expanded survival studies to a rabbit model. Optimum protection from SEB-induced lethality occurred around 4 days after total-body radiation exposure. Note the significant differences in LD₅₀ between the SEB-treated and irradiated, SEB-inoculated rabbits (Table III).

TABLE III. PERCENTAGE SURVIVAL OF DUTCH RABBITS GIVEN EITHER SEB, IM, OR IRRADIATED (500 R) AND GIVEN SEB IM

DOSE (μ g/kg)	% SURVIVAL (NO. SURVIVORS/TOTAL)	
	SEB alone	XRT ^a 4 days before SEB
5	100 (6/6)	--
10	20 (1/5)	100 (5/5)
50	20 (1/5)	100 (5/5)
100	20 (1/5)	80 (4/5)
500	0 (0/6)	40 (2/5)
1,000	--	60 (3/5)
5,000	--	17 (1/6)
LD ₅₀ at 7 days ^b	14.6 μ g/kg	754.5 μ g/kg

^aX-radiation-treated with 500 R.

^bMedian lethal dose at 7 days postinoculation with SEB, determined by probit analysis.

Both groups of rabbits given SEB developed fever with 2 hr after IM injection of SEB; however, irradiated, SEB-treated rabbits were better able to maintain the fever response throughout the critical period of SEB toxicity. Future studies will include head irradiation to attempt central modification of SEB toxicity.

Vascular clearance kinetics of VEE in monkeys.

Vascular clearance kinetics of ³²P-TC-83 have been studied in 18 monkeys. Neither a preexisting bacterial (S. typhimurium) nor viral (17-D yellow fever) infection altered vascular kinetics of radiolabeled TC-83 in either sublethally irradiated (400 R) or normal monkeys. Clearance kinetics were essentially the same by both methods of assay (e.g., radioactive count data and PFU of TC-83 performed in Vero cells). The results show that prior irradiation exposure complicated by infection does not delay virus clearance kinetics sufficiently to explain the prolonged viremias found in irradiated monkeys given TC-83 (see Annual Report, this work unit, FY 75).

Humoral antibody responses following irradiation and TC-83.

In previous Annual Reports, FY 1974 and 1975, it was established that the humoral immune response was not suppressed indefinitely by 400 R of total-body irradiation, but was delayed and even enhanced, with peak antibody responses appearing 3 wk or more after those found in vaccine control animals. The question to be answered: Did the antibody responses of the animals in the irradiated groups follow the typical IgM-IgG response as expected of the vaccine control monkeys? Antibody separations were accomplished by sucrose density gradient separation techniques. Although not complete, certain obvious trends occurred as shown by the available data in Table IV.

TABLE IV. GEOMETRIC MEAN SERUM-NEUTRALIZING ANTIBODY SUBCLASS TITERS AFTER IMMUNIZATION WITH TC-83

NO. OF MONKEYS	GROUP	ANTIBODY SUBCLASS	RECIPROCAL TITER BY DAY POSTVACCINATION					
			8	12	18	26	35	42
6	VC ^a	IgM	21	20	<u>94</u>	42	25	19
		IgG	12	63	<u>297</u>	<u>472</u>	<u>404</u>	<u>529</u>
3	R-1 ^b	IgM	10	11	12	24	<u>40</u>	14
		IgG	10	11	12	24	<u>160</u>	<u>320</u>
3	R-4 ^b	IgM	--	15	32	<u>40</u>	25	29
		IgG	--	19	14	<u>47</u>	<u>286</u>	<u>640</u>
								23
								<u>254</u>

^aVC = vaccinated control.

^bIrradiated either 1 or 4 days before vaccination.

The vaccine-control animals responded with a typical IgM-IgG response, as did monkeys in both the R-1 and R-4 groups. However, the IgM-IgG conversion was delayed or prolonged in monkeys from the irradiated groups, as shown by the underlined titers in Table IV.

Publications:

1. Hilmas, D. E., and R. O. Spertzel. 1975. Response of sublethally irradiated monkeys to a replicating antigen. Infect. Immun. 12:592-601.
2. Scott, S. K., P. C. Kosch, and D. E. Hilmas. 1976. Serum lactate dehydrogenase of normal, stressed and yellow fever virus-infected rhesus monkeys. Lab. Anim. Sci. 26:436-442.

LITERATURE CITED

1. Nathanson, N., and G. A. Cole. 1971. Immunosuppression: a means to assess the role of the immune response in acute virus infections. Fed. Proc. 30:1822-1830.

- Jmb/d 5E
2. Thind, I. S., and W. H. Price. 1969. The effect of cyclophosphamide treatment on experimental arbovirus infections. Am. J. Epidemiol. 90:62-68.
 3. Nahmias, A. J., M. S. Hirsch, J. H. Kramer, and F. A. Murphy. 1969. Effect of antithymocyte serum on herpesvirus hominis (type 1) infection in adult mice. Proc. Soc. Exp. Biol. Med. 132:696-698.
 4. Gochenour, Jr., W. S. 1972. The comparative pathology of Venezuelan encephalitis virus infection in selected animal hosts, p. 113-117. In Venezuelan Encephalitis, Publication 243, Pan American Health Organization, Washington, D. C.
 5. Nathanson, N., D. Goldblatt, I. S. Thind, M. Davis, and W. H. Price. 1966. Histological studies of the monkey neurovirulence of group B arboviruses. I. A semiquantitative grading scale. Am. J. Epidemiol. 82:359-381.
 6. Hirsch, M. S., and F. A. Murphy. 1967. Effects of antithymocyte serum on 17-D yellow fever infection in adult mice. Nature 216:179-180.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a DA OA6416	2. DATE OF SUMMARY ^a 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SURVEY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY ^b U	6. WORK SECURITY ^b U	7. REGADING ^b NA	8. DOD/NINST ^b NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES ^c a. PRIMARY 62760A	PROGRAM ELEMENT PROJECT NUMBER 3A762760A834	TASK AREA NUMBER 01		10. LEVEL OF SUM A. WORK UNIT 011		
11. TITLE (Punch line with Security Classification Code) ^d (U) Rapid electron microscopic assay for virus particles of diseases of military medical importance						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^e 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry						
13. START DATE 62 02	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house			
17. CONTRACT/GRAANT		18. RESOURCES ESTIMATE PRECEDING FISCAL YEAR 76		19. PROFESSIONAL MAN YRS CURRENT 77	20. FUNDS (in thousands) 61	
21. DATES/EFFECTIVE: b. NUMBER: NA		EXPIRATION:		22. PERFORMING ORGANIZATION NAME: Physical Sciences Division USAMRIID ADDRESS: Fort Detrick, MD 21701		
c. TYPE: d. KIND OF AWARD:		e. AMOUNT: f. CUM. AMT.		PRINCIPAL INVESTIGATOR (Punch line if U.S. Academic Institution) NAME: Buzzell, A. TELEPHONE: 301 663-7181 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME: POC:DA		
23. GENERAL USE Foreign intelligence considered		24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Punch individual paragraphs identified by number. Punch line with Security Classification Code.) 23 (U) To develop a rapid electron microscopic assay for virus particles in small samples of infected sera to make detection and identification possible at particle concentrations low enough for early diagnosis. Such an assay could be done in an hour or so, whereas days are required for the assay methods currently in use, and thus essentially useless for early detection of BW agents. 24 (U) The method is being adapted for use with Millipore filters with a view to increasing the sensitivity of the assay. 25 (U) 75 07 - 76 06 - Development is continuing on a rapid electron microscopic assay of virus particles for early diagnosis. The assay is modified from one described by Sharp in 1948, in which virus is concentrated by centrifugation onto sugar. To transfer virus from the agar to an electron microscopic grid for counting, a method has been devised which is simpler, requiring far less virus than before. Moreover, to facilitate viral identification, negative staining can be incorporated in the transfer step with damage minimal even for virus usually disrupted by negative staining. Using stains of different charge structural alterations have even been produced in a bacteriophage test virus described previously, revealing features of the structure not recognized before. Sensitivity of the assay could be increased further, enough for early diagnosis, by using Millipore filters in place of agar in a modified centrifugation step. Virus transfer from Millipores seemed erratic initially and negative staining was often poor. The trouble arose from surface tension effects however, and from air trapped in the filters. Procedures have been developed to avoid these difficulties and indications are that virus can be transferred from Millipores with staining as good as that possible with agar. Even tissue culture fluids can now be assayed, facilitating the study of animal viruses.				

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Aspects of Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 011: Rapid Electron Microscopic Assay for Virus Particles
of Diseases of Military Medical Importance

Background:

In 1949, Sharp¹ showed that virus could be concentrated and partially purified by sedimentation onto agar in the ultracentrifuge, allowing particles to be counted at concentrations as low as $10^6/\text{ml}$. Particles were retrieved for electron microscopy by trapping them in a collodion film, formed on the agar. This can damage the virus and negative staining can only be done in a subsequent step which can further disrupt the particles.²

A procedure has been devised for washing the particles off the agar, by placing it over a hole in Saran wrap stretched across a frame, with a Formvar film attached below. Negative staining can thus be done simultaneously, minimizing damage to the particles. The procedure can be done with Millipore filters as well, so that greater concentration may be possible in the ultracentrifuge, if particles can be funneled through an orifice onto the filter. Since gels can sediment and a seal would be needed at the orifice, agar could not be readily used this way.³

Progress:

Work on the problem of adapting the transfer procedure for use with Millipore filters has shown that erratic results obtained initially were principally due to air trapped in the filter. With air present the stain solution cannot readily flow down through the filter under gravity, to wash the virus free, nor can salts diffuse into the filter from the virus solution.

Early in the year it was discovered that air can be trapped at the surface of a Millipore even when floating on water. To avoid trapping air when a drop of virus solution is added, the Millipore must be kept inverted, since fluid recedes from the surface rapidly when the Millipore is turned upright. It was also difficult at first, with Millipoles inverted, to center them with respect to the Formvar film during virus transfer, since Millipoles are not transparent like agar. Procedures have now been devised, however, which make manipulations easy, even with the filter inverted.

Before these procedures had been completely worked out, the supply of filters was depleted. When new ones were ordered the "VF" grade originally

used was no longer available and the new "VS" filters behaved differently. Although fluid flow was better, staining was poor and virus transfer erratic, since the filters affected the particles adversely. The problem proved to be an excessive amount of detergent in the new filters. To remove detergent from the filtering surface, where it could weaken the Formvar film as well as degrade particles, soaking was necessary with the Millipores floating on water, filter side down. Removal of the detergent could be monitored by observing motion of the filters on a thin sheet of water; but results with virus transfer were still erratic, even with filters apparently wholly free of detergent. Also the quality of the negative staining was worse than before. Phosphotungstate failed to penetrate the filters and the silicotungstate was granular and very dense, frequently being more disruptive to the virus than the detergent had been. Since the stain caused film breakage too, apparently dissolving in the Formvar, it seemed likely that the dense stain was the anhydrous form, known to be soluble in organic materials.⁴

This suggested the presence of air in the filters, so a study was made of steps in the soaking procedure where introduction of air might have occurred. The first source of air proved to be the initial wetting process. When the filters were placed face down on water with the periphery exposed, wetting occurred across the top before water could penetrate from below, through the finer pores of the filtering surface. Entrapment of air could be avoided here simply by wetting the filters first on individual drops.

After soaking, the filters must be turned over. To be sure that the upper surface is wet, the filters are put on separate drops first and inverted, then touched down to the water surface again. The Millipore sinks and water should drain through it rapidly when it is lifted back to the surface. Drainage had been rather slow, consistently, so this source of air was not recognized until recently, when procedures were introduced to obviate the difficulty.

With this and other problems solved, results with coliphage T2L, a commercially available test virus, were greatly improved; and staining has been achieved with phosphotungstate as good as that previously possible only with silicotungstate. As described in the preceding report, it had seemed possible that the charge of -7 on phosphotungstate might make penetration difficult, preventing the use of stains above pH 5, where silicotungstate also begins to hydrolyze,⁴ increasing its charge from -4 to -8. With staining now possible at pH 7, work could proceed with the vaccine virus TC-83, which is more stable above pH 7 than below. Work with the virus in culture fluid can also now be done more readily than before. With air excluded, filters are not so readily clogged by protein, which also interfered with staining previously, as well as with transfer of the virus.

A manuscript entitled "Electron microscopic assay for virus particles for early diagnosis" is in preparation.

Publications:

None.

LITERATURE CITED

1. Sharp, D. G. 1949. Enumeration of virus particles by electron micrography. Proc. Soc. Exp. Biol. Med. 70:54-59.
2. Smith, K. O., and J. L. Melnick. 1962. A method for staining virus particles and identifying their nucleic acid type in the electron microscope. Virology 17:480-490.
3. Svedberg, T., and K. O. Pedersen. 1940. The Ultracentrifuge. Oxford University Press, Oxford, p. 29-33.
4. Tsigdinos, G. A. 1969. Heteropoly compounds of molybdenum and tungsten. Molybdenum Chemicals, Chemical Data Series, Bulletin Cd 612a (revised). Climax Molybdenum Company, Greenwich, CT.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION# DA OA6420	2. DATE OF SUMMARY 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SURVEY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY ^a U	6. WORK SECURITY ^b U	7. REGRADING ^c NA	8. DA DOD/N INSTN ^d NL	9. SPECIFIC DATA-CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: 62760A	PROGRAM ELEMENT	PROJECT NUMBER 3A762760A834		TASK AREA NUMBER 01	11. LEVEL OF SUM A. WORK UNIT 015	
12. CONTRIBUTING CARDS 114(e)(f)						
13. TITLE (Provide with Security Classification Code) ^e (U) Cardiovascular and cardiac electrophysiological effects of infections of military significance						
14. SCIENTIFIC AND TECHNOLOGICAL AREAS ^f 003500 Clinical medicine; 004900 Defense; 012900 Physiology						
15. START DATE 71 03	16. ESTIMATED COMPLETION DATE CONT	17. FUNDING AGENCY DA	18. PERFORMANCE METHOD C. In-house			
19. CONTRACT/GRAANT		20. RESOURCES ESTIMATE				
21. DATES/EFFECTIVE:		EXPIRATION:	22. PROFESSIONAL MAN YRS 1.0			
23. NUMBER: NA		FISCAL YEAR 76	24. FUNDS (in thousands) 72.7			
25. TYPE:		CURRENT 77	26. CUM. AMT. 1.0			
27. KIND OF AWARD:		28. PERFORMING ORGANIZATION				
29. RESPONSIBLE DOG ORGANIZATION		NAME: Animal Assessment Division USAMRIID				
NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		ADDRESS: Fort Detrick, MD 21701				
30. RESPONSIBLE INDIVIDUAL		PRINCIPAL INVESTIGATOR (Provide DOD ID if U.S. Academic institution) NAME: Pettit, G. W. TELEPHONE: 301 663-2238 SOCIAL SECURITY ACCOUNT NUMBER:				
NAME: Metzger, J. F. TELEPHONE: 301 663-2833		ASSOCIATE INVESTIGATORS NAME: Hilmas, D. E. NAME: Liu, C. T.				
31. GENERAL USE Foreign intelligence considered		POC:DA				
32. KEYWORDS (Provide each with Security Classification Code) (U) Myocardium; (U) Physiology; (U) Infectious diseases; (U) Military medicine; (U) Myocardial contractility; (U) Electrophysiology; (U) Cardiovascular; BW defense						
33. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Provide that of each with Security Classification Code.) 23 (U) Cardiovascular failure is the cause of death in many infectious diseases which pose a potential BW threat against military personnel. This work unit is designed to study cardiovascular, dynamic, and electrophysiological changes in selected infectious diseases to provide clearer insight into the mechanisms for these effects so that therapeutic regimens can be improved and hospitalization time decreased. 24 (U) Using conventional methods of quantitating cardiovascular function, determine baseline values and then measure changes induced by infections induced in rhesus monkeys. In addition, other cardiovascular parameters will be measured. 25 (U) 75 07 - 76 06 - To identify factors which may contribute to the pathogenesis of enterotoxemia and infections, plasma levels of myocardial depressant factor (MDF), kininogen, prekallikrein, endotoxin, and fibrin split products were assayed. Basal kininogen was 1.9 ± 0.23 micrograms/ml in humans ($n=3$) and 0.32 ± 0.03 in monkeys ($n=7$), respectively. In 4 monkeys 1 mg/kg staphylococcal enterotoxin B IV resulted in kinin-system activation as compared to controls. Occurrence of fibrin split products indicated that fibrinolysis was also activated. In 5 monkeys, viral infection (17-D yellow fever) and bacterial sepsis (<i>Salmonella typhimurium</i>) also caused activation of the kinin system. MDF in 4 control rabbits was 20.5 ± 1.9 U; 12 hr post-SEB it increased significantly to 44.3 ± 4.8 . In 1 monkey, there was a terminal increase in MDF; in another there was evidence of decreased myocardial contractility; this might be related to increased plasma MDF. Large doses of IV SEB caused absorption of endotoxin from the gut into plasma in 10 of 13 rabbits; only rabbits which developed endotoxemia died and plasma levels of endotoxin were quite high. Publications: J. Infect. Dis. 134:in press, 1976. Arch. Int. Physiol. Biochim. 84, in press, 1976.						

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 015: Cardiovascular and cardiac electrophysiological effects of infections of military significance

Background:

Myocardial depressant factor (MDF) is a low MW (800-1,000) polypeptide present in the plasma of animals and humans in shock; it decreases contractility of the heart and thereby contributes to the pathogenesis of shock. We have developed assays for MDF in order to investigate its role in the pathogenesis of SEB shock and severe infections.

Enteritis and pulmonary edema are the most consistent and severe pathological findings in monkeys given a lethal IV dose of SEB. Pulmonary edema is the more lethal of these lesions; hence, we are investigating mechanisms by which SEB would predispose toward its formation. Since endotoxemia results in pulmonary edema, and enteritis associated with SEB may allow absorption of endogenous endotoxin from the gut, we proposed to measure plasma endotoxin during SEB shock. Kinins, fibrin split products (FSP) and complement have been shown to be capable of causing pulmonary edema and suggested as causes of pulmonary edema during gram-negative sepsis; they are possible causes of the pulmonary edema seen after IV SEB. Assays have been developed to measure components of the kinin system (kininogen, kallikrein and prekallikrein), and collaborative arrangements (kininogen, kallikrein assays for endotoxin, FSP, and complement. These assays will enable further study of the pathophysiologic mechanisms of SEB shock or viral and bacterial infections.

Progress:

I. Assay for MDF.

Lefer's group¹ discovered and have extensively studied MDF, which is a low MW polypeptide present in the plasma of humans and animals in all types of shock. We have conducted studies to detect the presence or absence of this factor during SEB shock in New Zealand rabbits and rhesus monkeys.

MDF is assayed by applying plasma ultrafiltrates (filtered to exclude peptides with MW > 20,000 to an isolated papillary muscle and measuring any change in force of contraction; MDF activity is then expressed as % reduction in contractile force.¹ The various peptides (MW < 1,500) in the sample are chromatographed by applying a concentrated sample of plasma

ultrafiltrate into a Biogel P-2 polyacrylamide gel (200-400 mesh) column (90 x 1.5 cm) and collecting column eluates in fractions (5 ml/tube). These eluates are then bioassayed for MDF activity in order to localize the activity to particular fractions. Lefer¹ claims that MDF activity resides in a fraction eluted at 80-100 ml, but in the past he has inconsistently claimed that the activity resided in column eluates ranging from 80-150 ml. Wangensteen et al.² have shown that high concentrations of inorganic ions (Na^+ , K^+ , Cl^-) are the cause of MDF activity seen at elution volumes of about 125 ml. Thus, since published data were questionable, it was necessary to establish that MDF activity is independent of inorganic ions. In these experiments we found that the MDF activity in elution volumes from 115-125 ml was due to high concentrations of inorganic ions (Na^+ , K^+ , Cl^-); their concentrations in all other eluted volumes were the same as the physiological salt solution used for elution.

One MDF unit causes a 1% depression in developed tension of an isolated papillary muscle. In order to verify that MDF was eluted in the volume between 80 and 100 ml, we compared MDF activity and chromatographic profiles of plasma ultrafiltrates from a rabbit before and during hemorrhagic shock. MDF activity in pre-shock ultrafiltrate was 0, and in shock ultrafiltrate was 19 U. Average MDF activity in 5-ml fractions eluted between 80 and 95 ml was 6.8 ± 3.9 MDF U for ultrafiltrates from control rabbits, and 18.1 ± 2.3 U in ultrafiltrates from shocked rabbits; these differences were significant ($P < 0.05$). Column chromatography indicated an increased occurrence of peptide bonds, (OD, 230 nm) in fractions which contained higher MDF activity. Thus, we confirmed that, in our hands, hemorrhagic shock in rabbits increases plasma MDF activity as defined by Lefer¹ and that this increase in MDF activity is not an artifact of the chromatographic procedure.

We are in the process of standardizing a chemical assay (utilizing paper chromatography) for MDF. This is a modification of an assay originally developed by Barenholz et al.³ and involves measuring the density of a ninhydrin-developed spot which has been shown to be MDF; final perfection of this assay will obviate the need for assay of MDF by the difficult bioassay.

II. Assays for components of the kinin system.

In conjunction with our studies on the pathogenesis of infections and enterotoxemia, we have developed assays for components of the kinin system, including kallikrein, prekallikrein and kininogen.

Kallikrein is measured by quantitating the 1-tosylarginine methylesterase (TAME) activity of plasma; prekallikrein activity is measured by TAME activity of plasma after conversion of prekallikrein to kallikrein by kaolin.

Kininogen levels represent the amount of kinin which can be produced by treatment of plasma with kallikrein. The kallikrein we use is human urinary

kallikrein and the kinin generated is measured, using suitable standards, by bioassay on guinea pig ileum. Employing this assay, we found that kininogen level in human plasma is $1.9 \mu\text{g/ml}$, ± 0.23 ($n=3$), and in monkeys, $0.32 \mu\text{g/ml}$, ± 0.03 ($n=7$).

III. MDF, endotoxin, and kinins levels after IV SEB in rabbits and monkeys.

Pathologic findings from 6 monkeys given lethal doses of SEB revealed that enteritis and pulmonary edema were the most consistent and severe pathologic findings. Since endotoxemia results in pulmonary edema⁴ and enteritis may allow absorption of endogenous endotoxin from the gut, we measured plasma levels of endotoxin after IV SEB (800-1,000 $\mu\text{g/kg}$) in 13 rabbits. All samples taken before administration of endotoxin were negative, but 12 hr after administration of SEB, endotoxemia was detectable in the plasma of 10. Only rabbits which developed endotoxemia died, and their plasma levels of endotoxin were comparable to those detected by other workers after giving rabbits a lethal dose of IV endotoxin.

In other experiments, we measured MDF levels in plasma from rabbits ($n=3$) 11-12 hr post-SEB (1 mg/kg, IV) and in plasma from a group of suitable controls ($n=4$). The mean MDF level in rabbits that received SEB was 44.3 ± 4.8 U, which was significantly greater ($P < 0.05$) than the level in the control group (20.5 ± 1.9). These levels are consistent with those measured by others in shocked and nonshocked animals.¹

Kinins, FSP and complement have all been shown to be capable of causing pulmonary edema during gram-negative sepsis and are possible causes of the pulmonary edema seen after IV SEB intoxication. In order to investigate the role of these factors in the pathogenesis of enterotoxemia, experiments were performed on 4 monkeys before and after 1 mg/kg of SEB, IV (Tables I-II).

TABLE I. MEASUREMENTS OF BLOOD PRESSURE CHANGES IN 4 MONKEYS AFTER SEB.

BLOOD PRESSURES	NO. OF MEASUREMENTS/MONKEY			
	X-870	P-419	4965	T-10
Baseline	2	2	2	2
<u>Post-SEB</u>				
> 90% of baseline	0	1	3	0
70-90% of baseline	2	4	0	4
< 70% of baseline	3	3	1	2

TABLE II. DATA COMPARED WITH RESPECT TO BLOOD PRESSURE OF 4 MONKEYS.

PARAMETER	MEAN \pm SE (NO. OBSERVATIONS)			
	After SEB			
	Baseline	>90%	70-90%	<70%
CVP (cm H ₂ O)	-0.5 \pm 0.9 (6)	1 \pm 0.6 (4)	-1.8 \pm 1.7 (6)	0.2 \pm 0.9 (7)
HR (beats/min)	170 \pm 14 (6)	210 \pm 20 (4)	233 \pm 11 (6)	224 \pm 15 (7)
Skin temperature (°C)	35.5 \pm 0.3 (5)	35.5 \pm 0.4 (4)	35.5 \pm 0.4 (6)	34.3 \pm 0.7 (7)
Kallikrein Inhibitor (units)	0.75 \pm 0.09 (7)	0.86 \pm 0.01 (3)	0.79 \pm 0.07 (10)	0.68 \pm 0.09 (9)
Prekallikrein (μ mol/ml/hr ^a)	165.1 \pm 6.8 (7)	152.1 \pm 7.7 (4)	143.8 \pm 4.3 (10)	119.1 \pm 7.9 (9)
Kallikrein (μ mol/ml/hr)	8.3 \pm 0.8 (7)	7.6 \pm 0.8 (4)	7.7 \pm 0.7 (10)	7.3 \pm 0.8 (9)
FSP (μ g/ml)	0 (6)	2.5 \pm 2.2 (3)	14.0 \pm 5.5 (6)	12.9 \pm 2.2 (7)
Platelets (no. $\times 10^5$)	2.41 (2)	•	2.07 (2)	1.8 (2)
Hematocrit (%)	50 (2)		54 (2)	59 \pm 2 (3)
Myocardial depressant factor (PC units ^b)	0.13 (2)		0.16 (1)	0.20 \pm 0.05 (4)

^aMeasured as TAME-esterase activity.^bMeasured as paper chromatographic units.

The following physical parameters were measured: central venous pressure (CVP), mean arterial blood pressure (MABP), heart rate (HR) and skin temperature (ST). Plasma samples were withdrawn for assay of kininogen, MDF, kallikrein, kallikrein inhibitor, prekallikrein, endotoxin, total hemolytic complement (THC), and FSP. Since decreased MABP is the best indicator of shock, we have

tabulated our data in catagories determined by the decrease in MABP. Accordingly, in Table I we have catagorized our measurements of MABP. Two base-line measurements were taken for each monkey; then blood pressure measurements after SEB were placed in 1 of 3 catagories.

In Table II this system was utilized for tabulation of the other data. With results obtained thus far, we found that there is no apparent change in CVP or THC during the course of SEB toxemia. The appearance of FSP in plasma (Table II) is indicative of an increase in fibrinolytic activity. Changes in skin temperature were inconclusive. In the monkey for which data have been analyzed, MDF did not increase until immediately before death. Although MDF did not increase progressively during the course of this experiment, the increased level in samples where MABP was less than 70% of baseline suggests that MDF may have contributed to the demise of the animal. In these 4 monkeys, gradual prekallikrein depletion (Table II) indicated that kinin-system activation had occurred. These data suggest that kinin-system activation and terminal MDF production may be involved in the pathogenesis of SEB toxicity. Assay of samples for kininogen is in progress; kininogen depletion occurring coincident with prekallikrein depletion would lend more support to the statement that SEB causes activation of the kinin system.

In order to attempt to correlate changes in *in vivo* myocardial contractility with changes in plasma levels of MDF after IV SEB, a chaired monkey was instrumented for measurement at hourly intervals of left ventricular pressure (P) and an index of myocardial contractility, contractile element velocity at 40 mm Hg developed pressure ($V_{ce_{40}}$). MABP decreased from 100 to 50 mm Hg during the 15-hr period following injection of the SEB (0.1 mg/kg, IV). $V_{ce_{40}}$ varied little during the 3-day control period [1.20 ± 0.023 muscle lengths (ML)/sec], but was significantly increased ($P < 0.01$) during the first 8 hr following injection (1.99 ± 0.301 ML/sec) ($n=4$), then decreased significantly ($P < 0.01$) to 1.16 ± 0.04 ML/sec ($n=4$) from 9-14 hr. HR was 150 beats/min before injection of SEB and was about 250 beats/min at all times after injection of SEB. The fact that HR and $V_{ce_{40}}$ both increased during the first 8 hr indicated that sympathetic outflow was increased. Thus, HR stayed high throughout the post-SEB period, but $V_{ce_{40}}$ returned to control levels by 8 hr. This refractoriness to sympathetic stimulation indicated that myocardial depression may have occurred during the later stages of SEB shock. A number of factors may be responsible for this myocardial depression. Further work will be needed in order to correlate changes in plasma MDF activity with these changes in myocardial contractility.

IV. Experiments involving assessment of kinin-system activation during viral and bacterial infection.

In experiments with 3 monkeys, infection with 17-D vaccine strain of YF resulted in prekallikrein depletion (Table III) and increased kallikrein on day 6 postinfection. In experiments with 2 monkeys, infection with Salmonella typhimurium resulted in prekallikrein depletion and increased kallikrein.

Effects of radiation were inconclusive. These results indicate that viral infection (17-D YF) activates the kinin system, and confirm results obtained by others who have shown that bacterial sepsis causes kinin-system activation.

TABLE III. KININ SYSTEM WITH INFECTION AND IRRADIATION.

STUDY	MONKEY NO.	CONDITION	KALLIKREIN	PREKALLIKREIN	KININOGEN ^a
			μmol/ml/hr	(μg/ml)	
17-D YF	T-22	Control	7.5	201.8	
		6 da postinfection	11.8	151.8	
T-7		Control	10.4	234.5	
		1 da postinfection	10.2	240.7	
		6 da postinfection	11.0	180.7	2.46
T-1		Control	10.2	253.5	
		1 da postinfection	8.8	264.3	
		6 da postinfection	12.0	203.0	3.50
17-D YF + irradiation	X-960	Control	6.6	192.3	
		6 da postinfection, 4 da postirradiation	10.4	195.5	
<u>S. typhi-</u> <u>murium</u>	T-9	Control	6.0	156.1	
		2 da postinfection	10.5	137.3	2.20
T-6		Control	11.0	146.0	
		2 da postinfection, 4 da postirradiation	15	100.4	2.24
X-990		Control	8.7	193.9	
		No infection, no irradiation	9.0	173.2	
Controls	T-18	Control	11.5	207.8	
		4 da postirradiation, no infection	11.8	199.2	
X-983		Control	--	--	2.60
		4 da postirradiation, no infection	12.5	2.8	<2.00
X-987		Control	--	--	2.52
		No irradiation, no infection	10.5	204.5	2.1

^aTrypsin used as kinin-generating enzyme.

Publications:

1. Pettit, G. W., M. R. Elwell, and P. B. Jahrling. 1976. Possible endotoxemia in rabbits after intravenous injection of staphylococcal enterotoxin B. *J. Infect. Dis.* 134:in press.
2. Pettit, G. W., M. R. Elwell, R. T. Faulkner, and K. A. Bostian. 1976. Oral fructose tolerance, gastric emptying and absorption: a compartmental model. *Arch. Int. Physiol. Biochem.* 84:in press

LITERATURE CITED

1. Lefer, A. M., and J. A. Spath. 1974. Pancreatic hypoperfusion and the production of a myocardial depressant factor in hemorrhagic shock. *Ann. Surg.* 179:868-876.
2. Wangensteen, S. L., W. G. Ramey, W. W. Fergusen, and J. R. Stanling. 1973. Plasma myocardial depressant activity (shock factor) identified as salt in the cat papillary muscle bioassay system. *J. Trauma* 13:181-194.
3. Barenholz, Y., J. N. Leffler, and A. M. Lefer. 1973. Detection by chemical means of a myocardial depressant factor in plasma of animals in circulatory shock. *Isr. J. Med. Sci.* 9:640-647.
4. Clowes, G. H. A. 1974. Pulmonary abnormalities in sepsis. *Surg. Clin. North Am.* 54:993-1013.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ¹	2. DATE OF SUMMARY ²	REPORT CONTROL SYMBOL	
3. DATE PREV SURVEY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY ³ U	6. WORK SECURITY ⁴ U	7. REGARDING ⁵ NA	8. DISEN INSTN ⁶ NL	9. SPECIFIC DATA-CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT
10. NO./CODES: ⁷ a. PRIMARY b. CONTRIBUTING c. CONFIDENTIAL d. CONFIDENTIAL	PROGRAM ELEMENT 62760A	PROJECT NUMBER 3A762760A834		TASK AREA NUMBER 01		WORK UNIT NUMBER 020	
11. TITLE (Proceed with Security Classification Code) (U) Acute phase protein dynamics in the rat during infection							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁸ 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry							
13. START DATE 73 07	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house				
17. CONTRACT/GRANT a. DATES/EFFECTIVE: b. NUMBER: c. TYPE: d. KIND OF AWARD:		EXPIRATION: NA	18. RESOURCES ESTIMATE PRECEDING FISCAL YEAR 76 77	19. PROFESSIONAL MAN YRS 1.0 1.0	20. FUNDS IN HUNDREDS 119.0 52.0		
21. RESPONSIBLE DOD ORGANIZATION NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		22. PERFORMING ORGANIZATION NAME: Physical Sciences Division USAMRIID ADDRESS: Fort Detrick, MD 21701 PRINCIPAL INVESTIGATOR (Punch SEAN II U.S. Academic Institution) NAME: Abeles, F. B. TELEPHONE: 301 663-7181 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME: POC:DA					
23. KEYWORDS (Proceed EACH with Security Classification Code) (U) Rats; (U) Protein; (U) Infectious diseases; (U) Inflammation; (U) Military medicine; (U) BW defense; (U) Early detection; (U) Diagnosis							
24. TECHNICAL OBJECTIVE, ⁹ 25. APPROACH, 26. PROGRESS (Punch individual paragraphs identified by number. Proceed with Security Classification Code.) 23 (U) Investigate changes in acute-phase proteins during infections of unique military importance in BW defense. Provide information useful in the early detection of disease, diagnosis of specific diseases and inflammation, as well as provide techniques to control acute-phase protein synthesis so as to regulate the course of an infection and promote rapid recovery. 24 (U) Infect normal and surgically altered rats with a variety of organisms and measure acute-phase proteins and their kinetics. Study the synthesis and translation of acute-phase protein mRNA with in vitro protein synthesizing systems. 25 (U) 75 07 - 76 06 - Progress has been primarily in the development of in vitro protein synthesizing assay systems for mRNA. At the present time we have developed a system capable of translating rat liver mRNA. However problems still exist with regard to sensitivity and capability of translating all classes of mRNA.							
Available to contractors upon originator's approval.							

DD FORM 1 MAR 68 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

* U.S. GPO: 1974-840-843/8691

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Aspects of Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 020: Acute Phase Protein Dynamics in the Rat During Infection

Background:

An understanding is needed of the regulatory function of serum proteins secreted by the liver during infection and inflammation. The diseased state is characterized in part by changes in the concentration of certain serum proteins. These changes can be represented by increased levels of fibrinogen, C-reactive protein, α_1 -acid glycoprotein, α_1 -antitrypsin, α_2 -acute-phase globulin (rat), α_1 -acute-phase globulin (rat), ceruloplasmin, haptoglobin, and seromucoid, and decreased albumin.¹ The purpose of this study is to obtain information on the metabolism and function of these types of serum proteins in disease. Specifically most of the work has focused on the changes and physiology of α_2 -macroglobulin (α_2 -MFG). In the rat this acute-phase reactant increases \geq 100-fold from normal levels (which are barely detectable by radial immunoassay techniques). The work during the period of this report has dealt with the extraction and identification of the mRNA of acute-phase proteins and their translation of cell free protein synthesizing systems.

Progress:

Work during this period was devoted to developing a system capable of translating mRNA from rat liver. Three separate ribosome sources were evaluated as potential translating systems. According to the literature all of them are mRNA-dependent and should be capable of translating completed proteins from mRNA isolated from other cells.

The 3 systems were: (1) RNAase-treated rabbit reticulocyte lysates, (2) mouse ascitic tumor cells, and (3) wheat germ embryos. According to our experience both the rabbit reticulocyte and mouse ascitic cell system were incapable of translating liver mRNA. All attempts to show a liver mRNA-dependent incorporation of 3 H-leucine into protein were unsuccessful although these systems did respond as described to the artificial messenger, polyuridylic acid.

On the other hand we have consistently found that rat liver mRNA increased 3 H-leucine incorporation in trichloroacetic acid-precipitable proteins using ribosome wheat embryos. However, 2 problems have blocked

progress with this system. First, the increase in protein synthesis by mRNA is only 3-fold over controls without exogenously added mRNA. Secondly, we have observed little or no activity with mRNA isolated from polyribosomes associated with the endoplasmic reticulum. Supposedly, it is this class of polysomes that is actively associated with the production of serum proteins. Following protein synthesis by polysomes isolated directly from rat liver, we have also observed that protein synthesis by free polysomes is more active than that by membrane bound polyribosomes.

At this time, the reason for the inefficiency of mRNA from bound ribosomes is not known. At the present time we are trying alternative means for extracting and purifying bound mRNA with the hope of developing techniques which would minimize any destruction of the mRNA.

Publications:

None.

LITERATURE CITED

1. Koj, A. 1974. Acute-phase reactants. Their synthesis, turnover and biological significance. p. 73-132. In *Structure and Function of Plasma Proteins*. (ed. A. C. Allison) Plenum Press, New York.
2. Brawerman, G. 1974. The isolation of messenger RNA from mammalian cells. *Methods Enzymol.* 30:605-761.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA OF6412	2. DATE OF SUMMARY 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SURVEY 75 04 09	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGARING NA	8. DISTR INSTRN NL	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: a. PRIMARY b. CONTRIBUTING c. 114(e)(f)	PROGRAM ELEMENT 62760A	PROJECT NUMBER 3A762760A834	TASK AREA NUMBER 01	11. LEVEL OF SUM A WORK UNIT WORK UNIT NUMBER 022		
12. TITLE (Print in Security Classification Code) (U) Effect of infection on intermediary metabolism						
13. SCIENTIFIC AND TECHNOLOGICAL AREAS 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry						
14. START DATE 74 07	15. ESTIMATED COMPLETION DATE CONT	16. FUNDING AGENCY DA	17. PERFORMANCE METHOD C. In-house			
18. CONTRACT/GRAANT a. DATES/EFFECTIVE: b. NUMBER: c. TYPE: d. KIND OF AWARD:		19. RESOURCES ESTIMATE EXPIRATION: FISCAL YEAR	20. PROFESSIONAL MAN YRS 1.0	21. FUNDS (\$ thousands) 177		
		CURRENT 77	1.0	174		
22. RESPONSIBLE DOG ORGANIZATION NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		23. PERFORMING ORGANIZATION NAME: Physical Sciences Division USAMRIID ADDRESS: Fort Detrick, MD 21701				
RESPONSIBLE INDIVIDUAL NAME: Metrger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Print in Security Classification Code) NAME: Neufeld, H. A. TELEPHONE: 301 663-7181 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME:				
24. GENERAL USE Foreign intelligence considered		POC:DA				
25. TECHNICAL OBJECTIVE, 26. APPROACH, 27. PROGRESS (Print in Security Classification Code) 23 (U) To maintain by appropriate therapy the body stores which are utilized as a source of energy during infectious diseases of unique importance in military medicine and biological warfare. During periods of decreased food intake the body utilizes its own fat and protein to supply energy to various cells. Inability of the host to utilize its fat stores during infectious disease could explain the marked protein-wasting associated with illness. An understanding of these metabolic changes can lead to effective nutrient therapy which would reduce the protein-wasting and promote rapid recovery. 24 (U) Microanalytical methods for the study of various metabolites and their alterations caused by infection. 25 (U) 75 07 - 76 06 - All infections, median lethal doses of endotoxin, and turpentine abscess caused marked impairment of the normal fasting-induced ketosis in rats and guinea pigs. Perfusion studies using livers from infected animals showed an impairment in the ability of the liver to synthesize ketone bodies when presented with oleic acid, a fatty acid requiring carnitine for transport into the mitochondria. Mitochondria, however, when presented with palmitoyl carnitine were able to carry out the oxidative process. These observations, together with the observation that ketone body production is depressed in an infected diabetic rat points to a lesion at the liver site, perhaps represented by an infection-induced reduction of carnitine in the mitochondria and whole liver.						
Publications: Fed. Proc. 35:1502, 1976. Metabolism 25, in press, 1976.						

BODY OF REPORT

Project No. 3A762760-834: Medical Defense Aspects of Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 022: Effect of Infectious Disease on Intermediary Metabolism

Background:

Curnow, in unpublished reports from this laboratory, claimed that livers from infected rats had a marked impairment in the ability to carry on gluconeogenesis when such livers were perfused with pyruvate or alanine. A careful study of the conditions used by him suggested that many of the animals used were actually agonal, thus, the results were not typical of the progress of the infection prior to the agonal state; this has, indeed, been verified. Perfusion experiments and other experiments designed to measure gluconeogenesis found, not only no impairment due to infection, but perhaps, even stimulation. One approach to the study of gluconeogenesis was to measure the redox state of the cell. This was done by determining the NAD⁺:NADH ratio in the cytoplasm and mitochondria according to established techniques. The results of this study showed that there was no impairment in the redox state in the cytoplasm. The determination of the mitochondrial redox state was done by determining the concentrations of acetoacetate and β-hydroxybutyrate. It was found that infection caused a marked decrease in the hepatic concentration of these metabolites, in contrast to the normal fasted animal where their concentrations increased. Subsequent experiments proved that this decline was also apparent in the blood stream. It has been the purpose of this study to assess the magnitude of this effect, determine the mechanism, and assess the role of this lesion in the treatment of infection.

Progress:

Three pathogenic organisms were chosen as models in the rat: Streptococcus pneumoniae, Francisella tularensis, and Salmonella typhimurium. Each infection resulted in a marked inhibition of the fasting-induced ketosis. This lack of ketone bodies was demonstrable in the liver and in the blood and has now been verified in guinea pigs as well as rats. Moreover, if rats were fasted for 24 hr to induce a high level of circulating ketone bodies and then infected, the introduction of the infection caused a rapid decline in ketone body concentrations. This observation suggested that infection caused the induction of a metabolic lesion in one of the following areas: oxidation of fatty acids at the mitochondrial level, entrance of the fatty acid into the mitochondria, or mobilization of the fatty acid from fat depots in the body.

It was determined that when mitochondria from the infected rats livers were presented with fatty acids in the form of the carnitine ester (palmitoyl carnitine), there was no impairment in oxidation. Studies are underway in this division by another investigator to determine whether or not there exists a lesion at the fat pad.

It has been determined that the livers from infected animals, when perfused with oleic acid, have a decided impairment in their ability to convert the oleic acid to ketone bodies. This suggests, then, that the lesion lies at the site of the mitochondrial membrane and might involve carnitine. Studies on this hypothesis are now underway. There is also the possibility that there is a direct hormonal involvement with this infection-induced metabolic lesion. Two hormonal conditions have been investigated. Thyroidectomized animals, which have been infected, show the previously described inhibition of ketosis. It has been postulated, since there is a marked rise in portal insulin following infection, that insulin causes an inhibition of lipolysis, thus reducing the level of circulating free fatty acids which would thus result in a decreased production of ketone bodies. In one experiment, animals made diabetic by the administration of streptozotocin were infected with S. pneumoniae. After 48 hr there was dramatic reduction in the concentration of circulating ketone bodies in the infected diabetic animals. However, peripheral insulin values during an infection with S. pneumoniae are not dramatically altered. Thus, the data so far obtained seem to suggest that the primary lesion causing the inhibition of ketone body formation during infection is not directly influenced by thyroxine or insulin.

Very recent data have led to the concept that the metabolic response to stress may be dependent upon the type of stress imposed upon the animal. The inhibition of fasting-induced ketosis brought on by infection can also be caused by the administration of endotoxin or by the formation of a turpentine abscess. Imposition, however, of a nonseptic stress such as femoral fracture or screen-restraint caused no inhibition of fasting-induced ketosis. But all stress, septic and nonseptic alike, caused a reduction in the concentration of serum free fatty acids. Both of these stress responses differ from the well documented exercise stress in which there is an elevation both in free fatty acids and ketone bodies. In addition to this type of stress, the infection of a rat with VEE virus did not cause inhibition of ketosis. These data suggest, therefore, that any type of stress which induces the mobilization of leukocytes and the subsequent release of endogenous mediators (LEM) will cause inhibition of fasting-induced ketosis. Indeed, the induction of endogenous LEM by the IP injection of glycogen and heat-killed S. aureus caused an inhibition of ketosis.

Presentations:

1. Neufeld, H. A. The effect of bacterial infection on fasting-induced ketosis. Presented, Department of Medicine, Medical School, University of Virginia. July 1975.
2. Neufeld, H. A. The effect of bacterial infection on fasting-induced ketosis. Presented, Department of Biochemistry, School of Medicine, University of Rochester. March 1976.
3. Neufeld, H. A., M. V. Kaminski, J. A. Pace, and H. A. Stuhl. The effect of inflammatory and noninflammatory stress of ketosis in the rat. Presented, Annual Meeting of the American Society of Biological Chemists, San Francisco, CA, 6-10 June 1976. (Fed. Proc. 35: 1502, 1976).

Publication:

1. Neufeld, H. A., J. A. Pace, and F. E. White. 1976. The effect of bacterial infections on ketone concentrations in rat liver and blood and on free fatty acid concentrations in rat blood. *Metabolism* 25, in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ¹ DA OF6420	2. DATE OF SUMMARY ² 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUMMARY 75 05 12	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY ³ U	6. WORK SECURITY ⁴ U	7. REGRADING ⁵ NA	8. DISEN INSTRN ⁶ NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES ⁷ a. PRIMARY 62760A	PROGRAM ELEMENT PROJECT NUMBER 3A762760A834	TASK AREA NUMBER 01		WORK UNIT NUMBER 023		
b. CONTRIBUTING	c. EQUIPMENT CARDS 114(e)(f)					
11. TITLE (Pencile with Security Classification Code) (U) Pathophysiology of respiratory infections in animals						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁸ 003500 Clinical medicine; 004900 Defense; 002600 Biology (Pathology)						
13. START DATE 75 07	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house			
17. CONTRACT/GANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS 1.0	20. FUNDS (\$ thousands) 77.8	
21. DATES/EFFECTIVE: b. NUMBER: NA		FISCAL YEAR	CURRENT	22. PERFORMING ORGANIZATION NAME: Animal Assessment Division USAMRIID ADDRESS: Fort Detrick, MD 21701	23. GENERAL USE Foreign intelligence considered	
c. TYPE: d. AMOUNT: e. CUM. AMT.		76	77	24. SOCIAL SECURITY ACCOUNT NUMBER: NAME: Arensman, J. B. TELEPHONE: 301 663-2148 ASSOCIATE INVESTIGATORS NAME: Hilmas, D. E. NAME: Liu, C. T. POC:DA	25. TECHNICAL OBJECTIVE ⁹ 26. APPROACH. 27. PROGRESS (Pencile individual paragraphs identified by number. Pencile last of each with Security Classification Code.) 23 (U) Incapacitation resulting from respiratory infections and time required to regain normal pulmonary functions represent a very significant loss of military time and manpower. This work unit will define physiological mechanisms of pulmonary functional changes in selected diseases of medical importance in defense against BW agents, so that treatment regimens can be found which will reduce both lost time and losses of military manpower. 24 (U) Expose laboratory animals by the respiratory route to influenza virus or Klebsiella pneumoniae. Measure pulmonary functions using available or adapted known techniques. 25 (U) 75 07 - 76 06 - Studies were initiated into pathophysiology of influenza infections in the mouse model, and therapeutic effects of small particle aerosols (SPA) of rimantadine and ribavirin. After developing methodology for sampling arterial blood of mice, effects of influenza infections were evaluated. Results indicate that A2 influenza infection of mice is characterized by (1) severe hypoventilation (decreased partial pressure of oxygen and increased carbon dioxide (PCO-2)), compensated pulmonary acidosis (increased PCO-2 and carbonate and normal pH), (2) pulmonary edema (increased ratio of wet/dry lung weight), (3) hypothermia, and (4) reduced survival rates (35% or less). SPA of rimantadine (presented dose of 8 mg/kg/day for 4 days) initiated at 72 hr in infected mice significantly improved survival rates (85%) but failed to alter significantly the partial pressures of gases, respiratory acidosis, pulmonary edema, hypothermia, lung virus titers, or lung histopathology observed in untreated mice. SPA of ribavirin (presented dose of 40 mg/kg/day for 4 days) initiated at 6 hr significantly improved survival rates (95%). Pathophysiologic alterations were unchanged from those of normal noninfected control mice and lung virus titers and lung histopathology were significantly reduced when compared to untreated mice.	

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 023: The Pathophysiology of Respiratory Infections in Animals.

Background:

The A2 influenza model in mice and squirrel monkeys, and the Klebsiella pneumoniae model in rats have been established within USAMRIID.^{1,2} Since these infections are commonly observed in man, the existing animal models are suitable for studying mechanisms of viral and bacterial respiratory infection.

During acute viral and respiratory infections in man, it has been shown that there are (a) restrictive ventilatory defects, (b) increased alveolar-arterial oxygen tension gradient differences, and (c) airway obstruction. The first (a) are associated with decreased pulmonary compliance, increased airway resistance, and pathological alterations in the static and dynamic volumes of the lung. The second (b) differences are principally due to a decrease in the diffusion capacity of the lung, and/or a decrease in the effective ventilation and perfusion of the lung.

The total effect of these various pathologic alterations in lung function is to reduce the degree of oxygenation of arterial blood and, when severe, to promote CO₂ retention. Thus, one would expect tissue hypoxia and respiratory acidosis (due to this retention) to develop as the limitation of gas exchange is increased. Histopathologic studies of the lung indicate that pulmonary edema is a major component of these infectious processes.

Drugs effective in therapy against these infections may act by modifying the severity of O₂-CO₂ exchange defects and hence improve survival. Investigators³ have shown increased survival rates in rimantadine-treated, A2 influenza-infected mice. Rimantadine-treated, infected mice exhibited no detectable histopathologic or lung virus titer differences when compared with data from virus controls. This is in distinct contrast to results following aerosol therapy with ribavirin which markedly increased survival rates in A2 influenza-infected mice, and significantly reduced lung virus titers and histopathology. Therefore, are the increased survival rates observed with rimantadine-aerosol therapy of A2 influenza-infected mice mediated through physiological improvements in lung function?

Progress:

Mice were anesthetized using 1-2% halothane in moist room air. The abdominal cavity was opened. Arterial blood was sampled from the abdominal

aorta using a tuberculin syringe fitted with a 25-gauge needle, heparinized and analyzed within 10 min for pH and gas tensions. Normal values for blood gases, pH and bicarbonate in the mouse are presented in Table I.

In addition to blood sampling, determinations of rectal temperature and lung water content were conducted. With the exception of the lung wet:dry weight ratio, all measured parameters from virus control mice were significantly changed by day 3 from normal controls. On day 7, arterial PCO_2 , bicarbonate and wet:dry lung weight ratios were maximally increased, and arterial PO_2 and rectal temperatures were at their minimum. Arterial pH data were not significantly different from normal controls at any time. In the mouse influenza model, MTD was between 7 and 8 days postexposure, with few deaths before 6 or after 10 days.

These observed changes lead one to conclude that A2 influenza infection in the mouse model is characterized by (1) severe hypoventilation, as evidenced by severely depressed values for arterial PO_2 and increased values for PCO_2 ; (2) compensated pulmonary acidosis, a result of CO_2 retention with renal retention of bicarbonate as the principal compensating mechanism; (3) pulmonary edema, as evaluated by increased lung wet weights compared with their dry weights; and (4) hypothermia, most likely the result of a depressed metabolic rate subsequent to tissue hypoxia.

Studies were then initiated using rimantadine aerosol therapy. The drug was given by continuous SPA at a presented dose of 8 mg/kg/day. Therapy was initiated at 72 hr postchallenge and maintained for 4 days. Survival rates were 85% for therapy mice and 35% for virus controls. Rimantadine had no consistent effect on any parameter measured in the infected, drug-treated mice when compared to those of virus control mice (Table I). An earlier return toward normal values as evidenced by improved estimates of arterial PO_2 and PCO_2 and lower wet lung-weight ratios occurred from days 10-14 in treated mice. This earlier return towards normal values, however, does not explain improved survival observed during the period of maximum deaths at 6-9 days. This same tendency for an earlier return towards normal is observed with naturally occurring A2 influenza infections in man treated with amantadine, of which rimantadine is an analog.⁴

It appears that rimantadine does not induce detectable improvements in arterial PO_2 or PCO_2 . Alternative mechanisms may include, but are not limited to, the cardiovascular system and/or the CNS. Gross observations during the rimantadine experiment suggest that the status of the cardiovascular system may be improved. Support for a possible CNS effect comes from a report of CNS toxicity of the A2 influenza virus for mice.⁵ Also, remantadine is an analog of amantadine used in the treatment of Parkinson's disease.

TABLE I. EFFECTS OF SMALL-PARTICLE AEROSOLS OF RIMANTADINE AND RIBAVIRIN ON ARTERIAL PO₂; PCO₂, BICARBONATE, LUNG WATER CONTENT, AND RECTAL TEMPERATURE OF MICE INFECTED WITH A₂ INFLUENZA VIRUS

DAYS POST-EXPOSURE	GROUP ^a	PO ₂ (mm Hg)	PCO ₂ (mm Hg)	HCO ₃ (mM/L)	MEAN ± SE		Rectal Temperature (°C)
					Dry Lung Wt.	Wet Lung Wt.	
3	NC	97.2 ± 0.2 ^a	33.3 ± 1.5	17.6 ± 1.1	4.52 ± .11	38.3 ± 0.1	37.6 ± 0.1**
	VC	87.3 ± 3.1*	39.3 ± 1.1*	18.8 ± 0.5*	4.89 ± .10	36.8 ± 0.2*	
	Rimantadine ^b	90.6 ± 3.4	34.0 ± 1.0	16.5 ± 0.5	4.38 ± .09**	—	
	Ribavirin ^c	—	—	—	—	—	
5	NC	101.4 ± 2.5	30.5 ± 1.2	15.6 ± 0.6	4.82 ± .10	38.2 ± 0.2	34.2 ± 0.5*
	VC	60.7 ± 2.6*	42.6 ± 2.1*	22.2 ± 0.5*	6.31 ± .21*	34.5 ± 0.4	
	Rimantadine	64.0 ± 3.8	45.7 ± 1.8	23.7 ± 1.1	5.26 ± .18	34.5 ± 0.4	
	Ribavirin	86.0 ± 3.6**	31.8 ± 2.0**	16.6 ± 0.9**	4.85 ± .14**	38.3 ± 0.1**	
7	NC	102.5 ± 3.8	29.0 ± 0.5	16.2 ± 0.7	4.70 ± .15	39.0 ± 0.1	34.0 ± 1.0*
	VC	45.6 ± 6.4*	41.6 ± 1.4*	21.1 ± 1.9*	6.46 ± .40*	34.0 ± 1.0*	
	Rimantadine	58.3 ± 4.0	45.8 ± 4.2	20.4 ± 0.9	5.68 ± .29**	35.4 ± 0.3**	
	Ribavirin	86.3 ± 4.4**	33.2 ± 1.6**	15.9 ± 1.2**	4.90 ± .10**	38.1 ± 0.4**	
10	NC	96.8 ± 2.2	31.0 ± 1.8	18.1 ± 1.8	4.52 ± .16	38.5 ± 0.2	37.4 ± 0.7*
	VC	66.4 ± 13.8*	40.1 ± 4.2*	18.4 ± 1.2*	6.96 ± .84*	38.5 ± 0.3**	
	Rimantadine	69.8 ± 8.6	38.2 ± 2.8	17.9 ± 0.6	4.97 ± .10	38.5 ± 0.3**	
	Ribavirin	85.0 ± 3.3**	33.9 ± 1.1**	17.7 ± 0.6**	4.90 ± .10**	38.5 ± 0.1**	

*P<0.05 vs. NC

**P<0.05 vs. VC

^aNC=Normal controls; VC = Virus controls. All mice except NC were exposed to an approximate LD_{90/20} of A₂ influenza, mouse-adapted virus.

^bTherapy was initiated at 72 hr postexposure by continuous SPA for 4 days. Presented dose was approximately 8 mg/kg/day.

^cTherapy was initiated at 6 hr postexposure by intermittent SPA for 80 min/day for 4 days. Presented dose was approximately 40 mg/kg/day.

Experiments using ribavirin aerosol therapy, in A2 influenza-infected mice were also conducted. Ribavirin has been shown to produce very high rates (>95%) in A2 influenza-infected mice. Its apparent action is through antiviral effects, since lung virus titers and subsequent lung pathology are significantly reduced. In these studies, ribavirin therapy was initiated 6 hr postexposure and was administered for 80 min daily for 4 days. Therapy begun at 6 hr postexposure has been shown to be the most effective. Ribavirin therapy was highly effective in preventing the severe hypoventilation, respiratory acidosis, pulmonary edema, and hypothermia observed in untreated, infected mice (Table I). The minimal impairment of O₂ and CO₂ exchange, without pulmonary edema, in treated mice suggest that ventilation:perfusion imbalance is slight. These findings support the conclusion that the improved survival rates observed with ribavirin aerosol therapy are mediated through improvements in pulmonary function.

An experiment was also conducted to evaluate the effects of aerosolized rimantadine and ribavirin in normal mice. All parameters measured previously were repeated; no differences were observed between groups.

Publications:

None.

LITERATURE CITED

1. Berendt, R. F., G. G. Long, and J. S. Walker. 1975. Influenza alone and in sequence with pneumonia due to Streptococcus pneumoniae in the squirrel monkey. *J. Infect. Dis.* 132:689-693.
2. U.S. Army Medical Research Institute of Infectious Diseases. 1 July 1974. Annual Progress Report, FY 1974. p.47-56. Fort Detrick, Frederick, MD.
3. Stephen, E. L., J. W. Dominik, J. B. Moe, R. O. Spertzel, and J. S. Walker. 1975. Treatment of influenza infection of mice by using rimantadine hydrochlorides by the aerosol and intraperitoneal routes. *Antimicrob. Agents Chemother.* 8:154-158.
4. Little, J. W., R. G. Douglas, Jr., W. J. Hall, F. K. Roth, and D. Speers. 1976. Effect of amantadine on peripheral airways abnormalities in influenza A virus infections. Abstracts New York Academy of Sciences, 3rd Conference on Antiviral Substances, New York, 2-5 February 1976, Abstract no. 9.
5. Mims, C. A. 1960. An analysis of the toxicity for mice of influenza virus. I. Intracerebral toxicity. *Br. J. Exp. Pathol.* 41:586-592.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA OE6411	2. DATE OF SUMMARY 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SECY U	6. WORK SECURITY U	7. REGRADING NA	8. DISCHG INSTRN NL	9. SPECIFIC DATA CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT
10. NO./CODES: a. PRIMARY 62760A		PROGRAM ELEMENT PROJECT NUMBER 3A762760A834		TASK AREA NUMBER 01		WORK UNIT NUMBER 109	
b. CONTRIBUTING							
c. Information CARDS 114(e)(f)							
11. TITLE (Proceed with Security Classification Code) (U) Respiratory disease mechanisms and pathogenesis in airborne infections							
12. SCIENTIFIC AND TECHNOLOGICAL AREA 003500 Clinical medicine; 004900 Defense; 002600 Biology							
13. START DATE 73 02	14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-house		
17. CONTRACT/GRAANT a. DATES/EFFECTIVE: b. NUMBER: NA c. TYPE: d. KIND OF AWARD:				18. RESOURCES ESTIMATE PRECEDING FISCAL YEAR 76	19. PROFESSIONAL MAN YRS CURRENT 77	20. FUNDS (in thousands) 1.0	173.8
						1.0	192.0
19. RESPONSIBLE DOD ORGANIZATION NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701				20. PERFORMING ORGANIZATION NAME: Aerobiology Division USAMRIID ADDRESS: Fort Detrick, MD 21701			
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833				PRINCIPAL INVESTIGATOR (Provide DSN if U.S. Academic Institution) NAME: Larson, E. W. TELEPHONE: 301 663-7453 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME:			
21. GENERAL USE Foreign intelligence considered				POC:DA			
22. KEYWORD (Proceed with Security Classification Code) (U) Viral disease; (U) Aerosols; (U) Respiratory physiology; (U) Influenza virus; (U) Sendai virus; (U) Laboratory animals; (U) BW defense; (U) Military medicine							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Proceed text of each with Security Classification Code.) 23 (U) Examine respiratory disease mechanisms, including penetration, retention, clearance and replication of pathogens introduced in the host by enemy attack via the respiratory route. The research is essential for determining the pathogenesis and developing promising approaches to prophylaxis and therapy of infections, both respiratory and systemic, acquired by host inhalation, the most likely means of exposure in BW operations. 24 (U) Challenge experimental animal hosts with infectious microorganisms by 3 primary methods. Assess effects postexposure by quantitation from segments of the respiratory tract and peripheral organs.							
25 (U) 75 07 - 76 06 - A 5-parameter computer model developed for influenza proved equally valid for describing the Sendai virus population dynamics in the respiratory tissues of Sendai-infected mice. Successive alterations of the infections achieved with either antiviral therapy or the suppression of host-immunity demonstrated that the first 2 parameters of the model represent mathematical expressions of early virus replication properties. The third parameter defines the virus population limits and is determined mainly by the availability of host cell-receptor sites and a suitable host cell environment. The remaining parameters describe virus disappearance and represent mathematical expressions which define the influences of immunity in the host. Further work on the modeling of virus population dynamics will be discontinued with the completion of analytical studies now in progress.							
Publications: Infect. Immunity 13:438-447, 1976. Appl. Environ. Microbiol. 31:150-151, 1976							
* Available to contractors upon contractor's approval.							

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infections of Military Importance

Work Unit No. 834 01 109: Respiratory Disease Mechanisms and Pathogenesis
in Airborne Infections

Background:

The research under this work unit is directed to investigations on the pathogenesis of respiratory infections including the deposition and penetration properties of airborne microorganisms in the respiratory tract of experimental animals and the characterization of the microbial populations in respiratory tissues during infection. The working hypothesis is that the mathematical properties of the microbial population dynamics can provide insight into the fundamental relationships between factors of the host and the microorganism during infection.

The results of definitive investigations of influenza infections and initial studies with parainfluenza type 1, Sendai, virus in mice were reported previously.^{1,2} Studies this year were directed toward completing investigations with the Sendai model. The experimentation was designed to alter the infection characteristics by manipulating either the virus, by antiviral therapy, or the host, by the administration of immunosuppressants. It was reasoned that the relationships between the restrictions imposed on the experimental system and the effects observed in virus population dynamics would serve to identify specific mechanisms of the disease processes.

Progress:

The Sendai virus concentrations had been measured previously² in the respiratory tissues of mice as a function of time after small-particle aerosol (SPA) challenge with (1) unadapted virus in the absence of antiviral therapy, (2) mouse-adapted (lethal) virus without antiviral therapy, and (3) mouse-adapted virus with therapy. Therapy consisted of the continuous administration of ribavirin as SPA in the period from 24-72 hr after challenge.

Analytical studies directed toward modeling the dynamics of these virus populations revealed that the 5-parameter compartmental model developed for experimental influenza infections³ also provided adequate fits of the data observations with Sendai virus. Only the absolute values of the model parameters, or the time after challenge when virus-limiting factors became operative, changed with experimental treatment. These findings suggested that the fundamental determinants of virus population levels and, hence, the infectious processes were similar in both the influenza and Sendai systems.

Table I presents the model parameter values for the best fits of the model

TABLE I. COMPARTMENTAL MODEL VALUES FOR BEST FITS OF VIRUS POPULATIONS IN THE RESPIRATORY TISSUES OF SENDAI-INFECTED MICE

TREATMENT	TISSUE	PARAMETERS ^a					TIME-INTERRUPT ^b (hr)	LATENT PERIOD ^c (hr)
		P ₁	P ₂	P ₃	P ₄	P ₅		
Unadapted/ Untreated	Lung	-0.082	0.208	0.197	0.063	-0.001	24	5.2
	Trachea	-0.230	0.172	0.178	0.033	-0.001	24	7.2
	Nasopharynx	0.256	0.184	0.150	0.754	-0.002	24	4.0
Adapted/ Untreated	Lung	-0.940	0.071	0.044	0.011	-0.011	72	27.5
	Trachea	0.883	0.029	0.018	0.024	-0.010	72	4.0
	Nasopharynx	-0.587	0.049	0.050	0.005	-0.010	72	32.3
Adapted/ Treated	Lung	-3.59	0.086	0.074	0.350	-0.005	96	53.1
	Trachea	-2.09	0.054	0.101	0.003	-0.007	96	57.1
	Nasopharynx	-3.20	0.068	0.050	0.600	-0.010	96	61.9

^aP₁ = zero time intercept (\log_{10} EID₅₀), P₂ = slope of log-linear virus replication (\log_{10} EID₅₀/hr), P₃, P₄, P₅ = successive virus inhibition and disappearance parameters (\log_{10} EID₅₀/hr) operating from t-interrupt.

^bTime after challenge when log-linear virus replication was terminated.

^cTime after challenge when virus levels reached 1.0 \log_{10} EID₅₀/whole tissue.

to the Sendai virus populations in the respective respiratory tissues. Also indicated are (1) the time (latent period) after challenge when the virus concentrations first exceeded base-line levels and exponential virus replication commenced, and (2) the time when the virus-limiting parameters (P_3 , P_4 , P_5) of the model became operative, i.e., simple exponential increases in virus concentrations were interrupted leading to peaking and disappearance of virus from the tissues.

The model parameter values obtained when mice were challenged with unadapted virus were similar to those observed with influenza infections.³ Log-linear increase rates (P_2), approaching $0.2 \log_{10} \text{EID}_{50}/\text{hr}$ in all 3 respiratory tissues were high, but this exponential growth of the unadapted virus was terminated early following challenge (t -interrupt = 24 hr). A latent period in the range of 4-7 hr was consistent with that seen with influenza as were the values determined for the virus-limiting parameters (P_3 , P_4 , P_5).

Virus populations in mouse respiratory tissues following exposure to adapted virus developed at markedly slower rates and generally commenced later than with unadapted virus. However, exponential growth continued uninterrupted for longer periods of time; acceptable fits of the model were achieved only when the time-interrupt was extended to 72 hr in the case of untreated mice and 96 hr in ribavirin-treated mice. The apparent effect of this therapy was one of virustasis resulting in prolonged latent periods without any marked effects on the rates or durations of exponential virus growth. This time shift with the treatment reduced both the peak virus levels and the resident times of high virus concentrations in the respiratory tissues. The infections with adapted virus in the absence of ribavirin were uniformly lethal for mice with a MTD of 7.9 days. The survival among treated mice was 52% with a MTD of 9.6 days among mice that died.

Further analytical studies were performed to test the hypothesis that the first virus-limiting parameter (P_3) might be equal to the exponential virus growth rate (P_2). Recalculation of the model parameters with P_3 fixed at a level equal to P_2 yielded remarkable improvements in the coefficients of variation associated with the remaining model parameters. This finding suggested that the maximum virus levels in host respiratory tissues are most likely due to self-limiting factors which depend mainly on the availability of host cell receptor sites and a suitable host cell environment as opposed to the action of either host defense mechanisms or specific properties of the virus. These determinations and the observed effects of ribavirin therapy support a conclusion that model parameters P_1 and P_2 are primarily reflections of the virus properties per se and are generally exclusive of host defense factors.

Studies to investigate the effects of immunosuppression on virus population dynamics using the unadapted virus were conducted to determine the role of host immunity in limiting virus development and influencing virus disappearance during infection. Cyclophosphamide was administered SC

as the postchallenge immunosuppressant, while infected control mice received PBS. Virus concentrations measured in the respiratory tissues as a function of time after virus challenge are summarized for both the infected control and immunosuppressed mice in Table II.

The virus concentrations in the respiratory tissues of the infected controls were consistent with previous observations. Virus levels were high in all of the respiratory tissues by 48 hr and continued to increase at a reduced rate through 96 hr. By 168 hr the virus levels were reduced; by 264 hr all tissues were negative for virus. There were no deaths among infected control mice, which were caged separately for survival determinations.

High virus levels at 48 hr continued to increase at reduced rates through 216 hr in the immunosuppressed mice. These virus levels remained high through 264 hr and could not be determined after this time due to no survivors. These results were consistent with the hypothesis that factors in host immunity, while not the primary determinants of peak virus concentrations, are mainly responsible for virus disappearance.

Preliminary analytical studies have suggested that the compartmental model must be modified by eliminating parameters P_4 and P_5 in order to fit the virus population data obtained from the immunosuppressed mice. This indicates that these parameters may be regarded as mathematical expressions of the influence of host-immunity factors. The failure of immunosuppression to markedly affect the first virus-limiting parameter (P_3) further supports the earlier evidence presented that the maximum virus levels in host respiratory tissues are mainly determined by self-limiting viral factors and not host immunity.

Further work with Sendai virus and the compartmental modeling of virus population dynamics will be discontinued upon completion of the current analytical studies. Immediate efforts will be directed to the preparation of manuscripts to record the findings and show the relationships between the operative factors in the biological systems and the characteristics of the compartmental model. Future laboratory studies under this work unit will be directed toward investigations on the pathogenesis of Japanese B encephalitis as a respiratory model of potential BW importance.

TABLE II. WHOLE TISSUE CONCENTRATIONS OF UNADAPTED SENDAI VIRUS IN THE RESPIRATORY TISSUES OF IMMUNOSUPPRESSED AND INFECTED CONTROL MICE (N=3)

HOURS POSTCHALLENGE	MEAN LOG ₁₀ EID ₅₀ /WHOLE TISSUE ^a					
	Lung		Trachea		Nasopharynx	
	Control	Suppressed	Control	Suppressed	Control	Suppressed
48	5.34	5.85	5.50	5.43	5.21	4.71
96	6.22	6.13	6.22	6.18	5.94	6.68
168	2.22 ^b	6.82	0	5.57	4.97	6.82
216	1.94 ^b	8.19	0	6.95	3.41	7.20
264	0 ^c	5.69	0	6.13	0	6.02

^aMean virus levels of positive mice only.

^bN=1.

^c0<~1.10.

Publications:

1. Larson, E. W., J. W. Dominik, A. H. Rowberg, and G. A. Higbee. 1976. Influenza virus population dynamics in the respiratory tract of experimentally infected mice. *Infect. Immun.* 13:438-447.
2. Larson, E. W., H. W. Young, and J. S. Walker. 1976. Aerosol evaluations of the DeVilbiss No. 40 and Vaponefrin nebulizers. *Appl. Environ. Microbiol.* 31:150-151.

LITERATURE CITED

1. U.S. Army Medical Research Institute of Infectious Diseases. 1 July 1974. Annual Progress Report, FY 1974, pp. 93-102. Fort Detrick, Frederick, MD.
2. U.S. Army Medical Research Institute of Infectious Diseases. 1 July 1975. Annual Progress Report, FY 1975, pp. 61-67. Fort Detrick, Frederick, MD.
3. Larson, E. W., J. W. Dominik, A. H. Rowberg, and G. A. Higbee. 1976. Influenza virus population dynamics in the respiratory tract of experimentally infected mice. *Infect. Immun.* 13:438-447.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ¹ DA OE6420	2. DATE OF SUMMARY ² 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SURVEY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY. U	6. WORK SECURITY U	7. REGRADING ³ NA	8. DGSR/N INSTR'N NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES: ⁴ a. PRIMARY 62760A	PROGRAM ELEMENT PROJECT NUMBER 3A762760A834	TASK AREA NUMBER 01		WORK UNIT NUMBER 110			
c. CONTRIBUTING CARDS 114(e)(f)							
11. TITLE (Print job with Security Classification Code) (U) Mechanisms of renal response during toxemias and infectious diseases							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁵ 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry							
13. START DATE 73 08	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house				
17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS			
a. DATES/EFFECTIVE: EXPIRATION:		FISCAL YEAR	76	1.0			254.0
b. NUMBER: NA		CURRENT	77	1.0			289.0
c. TYPE: NA		d. AMOUNT: e. CUM. AMT.		b. FUNDS (In thousands)			
g. KIND OF AWARD: NA							
19. RESPONSIBLE DOO ORGANIZATION		NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		20. PERFORMING ORGANIZATION		NAME: Animal Assessment Division USAMRIID ADDRESS: Fort Detrick, MD 21701	
RESPONSIBLE INDIVIDUAL		NAME: Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Printed Name & U.S. Academic Institution) NAME: Liu, C. T. TELEPHONE: 301 663-2148 SOCIAL SECURITY ACCOUNT NUMBER: NAME: ASSOCIATE INVESTIGATORS NAME: POC:DA			
21. GENERAL USE		Foreign intelligence considered					
22. KEYWORD INDEX (Print job with Security Classification Code) (U) Renal function; (U) Staphylococcal enterotoxin B (SEB); (U) Military medicine; (U) Rocky Mountain spotted fever (RMSF); (U) Cardiovascular responses; (U) BW defense							
23. TECHNICAL OBJECTIVE. ⁶ 24. APPROACH. 25. PROGRESS (Print job with Security Classification Code) (U) To evaluate treatment of SEB-induced toxemia by measuring gastrointestinal motility, absorption, secretion, splanchnic circulation, and liver functions in animal models. This information is essential for understanding the mechanisms of SEB-induced gastrointestinal syndromes (vomiting and diarrhea) and for finding the most effective means for its treatment and prevention. Staphylococcal enterotoxemia presents formidable problems in military medical facilities and in BW defense.							
24. (U) Initially, examine SEB distribution and clearance, expanding on previous studies at this Institute.							
25. (U) 75 07 - 76 06 - Although severe cardiohepatic and renal depression was determined in IV SEB-challenged rhesus monkeys, supporting evidence for pulmonary dysfunction in the cause of death was provided. Positive-pressure breathing appeared to be effective for treatment of SEB-induced pulmonary edema aided by fluid administration. Pretreatment with total-body x-irradiation (400-500 R) was shown to prolong survival time of SEB-inoculated rhesus monkeys and Dutch rabbits.							
RMSF in rhesus monkeys was characterized by increased total body water, as well as plasma, RBC and blood volumes at 8 days. Although muscle and lung showed intracellular dehydration with loss of cellular Na and K ions, cellular overhydration with decreases in these extracellular ions was observed in the liver. The renal cortex demonstrated a decrease in levels of cellular sodium.							
Publications: The Physiologist 18:295, 1975. J. Appl. Physiol. 40:101-104, 1976. Am. J. Vet. Res. 37:969-974, 1976. J. Med. Primatol. 5: 2 papers, in press, 1976.							

Available to contractors upon contractor's own responsibility.

DD FORM 1 MAR 68 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

* U.S. GPO: 1974-840-843/8691

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 110: Mechanisms of Renal Responses during Toxemias and Infectious Diseases

Background:

I. Studies on SEB.

The general background for SEB studies has been presented in Annual Reports of FY 74 and 75. Although the exact cause of death in IV challenged monkeys is unknown, others^{1,2} demonstrated pulmonary edema with grossly increased lung weights during enterotoxemia, suggesting that pulmonary dysfunction may play a major role. Our aims were to study respiratory and circulatory responses to IV SEB shortly before death. In addition, water contents and electrolyte concentrations of various tissues were determined.

II. Studies on RMSF.

Prominent clinical signs and symptoms of RMSF in adults and children include persistent fever, headache, vasculitis, skin rash, edema, confusion, and coma. Harrell and Aikawa³ and Harrell⁴ emphasized that peripheral vascular collapse, capillary dilatation and blood pooling were induced during the early phase (1st wk) of RMSF. As proliferative and thrombocytic lesions developed in small vessels, hypoxia occurred in the areas supplied by the impaired circulation. Consequently, tissue necrosis and increase in capillary permeability were produced with losses of plasma water, electrolytes and proteins, as well as RBC. During peak illness, an increase in "thiocyanate space" was also shown.^{3,4}

Since rhesus monkeys develop a similar syndrome to human patients,⁵ this primate model was selected for studying mechanisms of certain RMSF-induced physiologic and biochemical changes.

Progress:

I. SEB studies.

Cardiohepatic changes 7-14 hr after IV SEB.

Using the same monkey as its own control (n=8), cardiohepatic responses to IV SEB (1 mg/kg), were studied 7-14 hr after injection. Mean blood pressure decreased from 126 to 94 mm Hg 14 hr after challenge. Cardiac output, stroke volume, cardiac work, and mean cardiac power continued to

decrease at 7 and 9 hr and increased slightly between 13 and 14 hr; however, the increased values were still below control levels. Both heart rate and total peripheral resistance increased and reached a peak at 8-9 hr and 7-8 hr after SEB, respectively. These values were reduced from their peak levels throughout the remainder of the experiment. Although the mean transit time from the inferior vena cava to dorsal aorta increased, central blood volume decreased. Liver functions were impaired, as shown by a prolonged half-life and slower disappearance rate of injected cardiolgreen dye.

Results from these SEB experiments indicate that: (a) mean arterial blood pressure was well maintained above 94 mm Hg during a 14-hr study, but decreased drastically just prior to death; (b) decreases in cardiac function reached a peak between 8 and 9 hr, while monkeys were still alive at that time, which suggested that decreased cardiac performance probably was not the major killing factor.

Renal changes 6-11 hr after IV SEB.

Renal changes between 6 and 11 hr after IV SEB (1 mg/kg) were studied in 10 rhesus monkeys. Although macaques were approaching death at 11 hr, the average mean blood pressure was still > 80 mm Hg. All renal functions were significantly decreased except that renal resistance was increased. Renal O₂ consumption decreased at 8 hr, but increased slightly between 10 and 11 hr.

Respiratory responses to IV SEB.

Techniques have been established for measuring pulmonary functions in conscious and anesthetized rhesus monkeys. Ketamine sedation (25 mg/kg) followed by an IV infusion of Na pentobarbital (0.05 mg/min) were used for anesthesia. Values for respiratory parameters were relatively constant in anesthetized monkeys. Effects of IV SEB on various pulmonary functions were also studied. Results from 4 monkeys are summarized in Table I. During the period of 6-11 hr after SEB, physiological dead space, and the respiratory quotient (RQ) increased, while airway resistance, functional residual capacity (FRC), CO₂ output and O₂ consumption decreased. Further, the surface tension of the lung extract from the SEB-inoculated monkey was increased, which indicated that pulmonary surfactant contents were reduced or that the lung may have collapsed slightly. The assumption of slight lung collapse and fluid accumulation was further supported by continuous decreases in FRC values, despite a simultaneous reduction in tidal volume 11 hr. The development of pulmonary edema may contribute to death during enterotoxemia in monkeys.

Effects of SEB in x-irradiated rhesus monkeys and Dutch rabbits.

It was found that total-body x-irradiation (400 R for monkeys, 500 R for rabbits) increased survival from SEB toxicity. For example, the

TABLE 1. RESPIRATORY RESPONSES TO IV SEB (1 mg/kg) IN 4 ANESTHETIZED MONKEYS.

PARAMETER	MEAN VALUE \pm SE BY HOURS		
	6	8	10
Tidal volume (ml)	32.0 \pm 3.5	39.2 \pm 6.9	44.8 \pm 7.4
Respiratory rate (cycle/min)	35.9 \pm 2.3	38.3 \pm 3.0	35.3 \pm 2.5
Physiological dead space (ml)	2.25 \pm 1.65	10.0 \pm 4.0	10.5 \pm 3.8
Dynamic compliance (ml/cm H ₂ O)	9.4 \pm 1.9	8.1 \pm 1.5	8.2 \pm 0.6
Airway resistance (cm H ₂ O/L/sec)	31.8 \pm 4.4	24.5 \pm 3.1	25.0 \pm 5.1
Air flow rate expired (ml/sec)	62.9 \pm 7.6	71.9 \pm 9.4	73.3 \pm 8.4
Air flow rate inspired (ml/sec)	48.8 \pm 4.0	56.8 \pm 5.8	72.0 \pm 8.7
Intraesophageal pressure (cm H ₂ O)	4.6 \pm .6	6.4 \pm 1.4	6.3 \pm 1.0
FRC ^a (ml)	96.3 \pm 4.2	83.4 \pm 3.9	60.9 \pm 2.6
CO ₂ output (L/hr/m ²)	8.67 \pm .12	8.32 \pm 0.25	7.24 \pm .24
O ₂ consumption (L/hr/m ²)	8.81 \pm .57	6.85 \pm 0.25	7.00 \pm .78
RQ	0.814 \pm .132	1.23 \pm 0.05	1.17 \pm 0.11
			0.896 \pm .039

x-irradiated monkey lived for 90 hr, compared to 15-20 hr after SEB injection (50 µg/kg) in nonirradiated monkeys. Although the magnitude of SEB-induced renal depression was not significantly different between control and irradiated monkeys, vasodilatation and unaltered cardiac output were demonstrated in the latter. Improvement of the cardiovascular system might account for the longer survival in monkeys pretreated with total-body x-irradiation. The following postulated mechanisms may also be responsible for alteration of SEB toxicity: (a) rate of cellular destruction or renal excretory rate of SEB may be increased and (b) substances produced through cellular repair from irradiation injury may antagonize SEB.

Significant prevention of SEB-induced death by pretreatment of total-body x-irradiation (500 R) has also been demonstrated in Dutch rabbits (see Work Unit 834 01 010). Survival appeared to correlate with the ability to maintain fever after IM injection of SEB (Table II). Sustained fever following SEB challenge may imply that SEB-induced fever in the irradiated host may originate centrally due to modification of hypothalamus functions, or that some SEB molecules may be destroyed by the increased metabolic rate (fever).

TABLE II. EFFECT OF SEB ON RECTAL TEMPERATURE ($^{\circ}$ C)
CHANGES IN NORMAL AND X-IRRADIATED DUTCH
RABBITS.

HOUR	TEMPERATURE $^{\circ}$ C	
	Control (n=6)	X-irradiated (n=6) 3 days prior to SEB
0	39.2 \pm 0.2	39.2 \pm 0.2
2	40.1 \pm 0.3	39.8 \pm 0.2
6	37.9 \pm 0.3	39.7 \pm 0.4
7	37.8 \pm 0.3	40.1 \pm 0.3
8	37.8 \pm 0.6	40.3 \pm 0.3
9	38.2 \pm 0.6	40.3 \pm 0.3
10	38.9 \pm 0.6	40.2 \pm 0.5
20	Dead	39.6 \pm 0.2 ^a

^an=3, 3 had died between 10 and 20 hr.

Effects of SEB on tissue water and electrolytes.

At the end of respiratory studies (11 hr after SEB), anesthetized monkeys were sacrificed and 14 different tissues from each monkey were taken. Benson's equations were used for calculation of intra- and extracellular water and electrolyte distributions. Normal control monkeys were anesthetized the same length of time and treated the same. Preliminary results are presented in Tables III - V. The following trends were revealed: (a) total water contents in gastrocnemius muscle and lung increased; (b) increased

TABLE III. EFFECT OF IV SEB (1 mg/kg) ON TISSUE WATER AND ELECTROLYTES IN CONTROL (n=3) AND SEB (n=4) MONKEYS.

Tissue	Group	H_2O (cc/kg FFWT ^a)		
		T ^b	E ^c	I ^d
Gastrocnemius muscle	Control	763 \pm 4	116 \pm 8	649 \pm 11
	SEB	782 \pm 2	163 \pm 44 ^e	619 \pm 35 ^e
Diaphragm	Control	764 \pm 7	218 \pm 19	546 \pm 26
	SEB	774 \pm 10	144 \pm 16 ^e	638 \pm 10 ^e
Lung	Control	802 \pm 8	325 ^f	484 ^f
	SEB	844 \pm 15	497 \pm 27 ^e	353 \pm 18 ^e
Heart	Control	811 \pm 5	274 \pm 39	537 \pm 42
	SEB	825 \pm 10	228 \pm 39	597 \pm 46
Renal cortex	Control	825 \pm 9	341 \pm 60	484 \pm 63
	SEB	835 \pm 7	365 \pm 69	470 \pm 69
Outer medulla	Control	846 \pm 13	237 ^f	622 ^f
	SEB	846 \pm 15	467 \pm 50	373 \pm 34
Inner medulla	Control	831 \pm 16	360 \pm 49 ^e	471 \pm 64
	SEB	854 \pm 16	554 \pm 16 ^e	300 \pm 9 ^e

^aFFWT = Fat-free wet tissue.

^bT = mEq total electrolytes/kg FFWT.

^cE = mEq extracellular electrolytes/kg extracellular H_2O .

^dI = mEq intracellular electrolytes/kg intracellular H_2O .

^eN = 3.

^fN = 2.

TABLE IV. EFFECT OF IV SEB (1 mg/kg) ON TISSUE WATER AND ELECTROLYTES OF CONTROL (n=3) AND SEB MONKEYS (n=4).

TISSUE	GROUP	ELECTROLYTES (mEq/kg FFWTa)					
		T _b	K ⁺	C _i ⁻	Na ⁺	K ⁺	I _d
Gastro-	Control	27 ± 2	107 ± 13	17 ± 2	15 ± 1	0.38 ± 0.01	17 ± 3
	SEB	43 ± 10	110 ± 13e	26 ± 6	22 ± 6e	0.55 ± 0.19	31 ± 15
Diaphragm	Control	39 ± 1	102 ± 2	30 ± 3	30 ± 3	0.72 ± 0.07	16 ± 3
	SEB	46 ± 4	89 ± 3	22 ± 3	19 ± 2e	0.48 ± 0.13e	36 ± 10e
Lung	Control	80 ± 3	65 ± 6	52 ± 7	44f	1.00f	60f
	SEB	81 ± 3	62 ± 3	63 ± 4	67 ± 4e	1.64 ± 0.22e	28 ± 5e
Heart	Control	46 ± 3	96 ± 4	36 ± 3	37 ± 6	0.93 ± 0.21	9 ± 6
	SEB	42 ± 5	99 ± 2	28 ± 4	31 ± 6	0.81 ± 0.27	22 ± 4
Renal cortex	Control	62 ± 5	86 ± 10	45 ± 5	46 ± 9	1.16 ± 0.29	35 ± 17
	SEB	61 ± 2	72 ± 5	48 ± 7	42 ± 10e	1.22 ± 0.25e	28 ± 13e
Outer medulla	Control	80 ± 6	78 ± 5	40 ± 7	32f	0.73f	95f
	SEB	69 ± 5	75 ± 2	53 ± 11	56 ± 12e	1.27 ± 0.30e	46 ± 4e
Inner medulla	Control	55 ± 2	68 ± 4	47 ± 5	38f	1.22 ± 0.25	33f
	SEB	68 ± 3	86 ± 8	67 ± 2e	66f	2.08 ± 0.07e	22f
^a FFWT = Fat-free wet tissue.							

b_T = mEq total electrolytes/kg FFWT.

c_E = mEq extracellular electrolytes/kg FFWT.

d_I = mEq intracellular electrolytes/kg intracellular H₂O.

e_N = 3.

f_N = 2.

TABLE V. EFFECT OF IV SEB (1 mg/kg) ON TISSUE WATER AND ELECTROLYTES.

Tissue	Group (Number of monkeys)	H ₂ O (cc/kg FFWT ^a)		Electrolytes mEq/kg FFWT					
		T	E _e	Na ⁺	T _b	K ⁺	Cl ⁻	E _c	Na ⁺
Brain	Control (3)	855	79	776	63	104	12	12	0.3
	SEB (1)	851	483	368	58	122	20	70	1.9
Brain medulla	Control (3)	951	230	721	58	109	30	33	0.9
	SEB (1)	822	152	670	77	87	58	22	0.6
Thalamus + hypo- thalamus	Control (3)	887	222	665	47	105	29	32	0.9
	SEB (1)	780	189	591	49	116	22	27	0.7
Cere- bellum	Control (3)	824	254	570	47	111	33	37	1.0
	SEB (1)	849	102	747	48	126	15	15	0.4
Spinal cord	Control (3)	885	440	445	67	96	55	64	1.6
	SEB (1)	797	190	607	76	107	25	27	0.8
Liver	Control (3)	781 ± 20	334 ^f	459 ^f	36 ± 7	96 ± 3	39 ± 9	--	--
	SEB (4)	782 ± 11	333 ± 70	439 ± 678	30 ± 68	82 ± 148	43 ± 98	--	--
Skin	Control (1)	806	--	--	126	39	99	--	--
	SEB (1)	716	--	--	106	32	86	--	--

^aFFWT = Fat-free wet tissue.^bT = mEq total electrolytes/kg FFWT.^cE = mEq extracellular electrolytes/kg extracellular H₂O.^dI = mEq intracellular electrolytes/kg intracellular H₂O.^eE = mEq extracellular electrolytes/kg FFWT.^fN = 2.^gN = 3.

extracellular water and decreased intracellular water occurred in skeletal muscle, lung, renal, outer medulla, and renal inner medulla; (c) within the central nervous system of normal monkeys, spinal cord and medulla oblongata showed the highest water, Na^+ and Cl^- contents, while cerebellum revealed the lowest values for water and Na^+ , which was also low in the thalamus and hypothalamus regions; and (d) an increased Na^+ concentration gradient was found in the normal monkey kidney between renal cortex and outer medulla, but this ionic gradient was less pronounced in kidneys of SEB-inoculated monkeys.

Treatment of SEB toxicity by positive-pressure breathing and fluid infusion.

Since severe dehydration and pulmonary edema were present during enterotoxemia in rhesus monkeys, positive pressure breathing (2-3 cm H_2O) and IV fluid infusion were applied for correcting these symptoms. Results are summarized in Table VI. This physiological approach to treatment seemed to prolong survival in 3 and prevented death in 1 monkey after SEB intoxication. Since the capillary permeability is increased after SEB, fluid therapy alone was not effective due to further accumulation of water in the lung. When lung tissue pressure is increased by positive-pressure breathing, increased tissue pressure opposes the accumulation of fluid out of alveolar capillary membranes. It appears that if pulmonary edema fails to occur after SEB inoculation, the monkey survives. The possibility may exist that some SEB molecules have been destroyed or metabolized reaching a sublethal dose level after a prolonged period of treatment.

TABLE VI. EFFECTS OF POSITIVE PRESSURE BREATHING AND FLUID INFUSION ON SURVIVAL TIME IN IV SEB-CHALLENGED MONKEYS.^a

MONKEY NO.	TIME OF TREATMENT AFTER SEB (hr)	FLUID ADMINISTERED	DURATION OF TREATMENT (Days)	SURVIVAL TIME (hr)
X-999	5	5% glucose in H_2O	3.5	Indefinitely
X-297 ^b	5	5% glucose in H_2O	0.5	12
P-709	2	5% glucose in 0.45% saline	2.0	72
P-710	2	5% glucose in lactated Ringer's	2.2	52

^aPositive pressure breathing (2-3 cm H_2O). Dose of SEB (50 $\mu\text{g}/\text{kg}$). Fluid infusion rate = 0.2 - 0.3 ml/min.

^bThis monkey appeared weak and not alert prior to SEB injection.

II. RMSF Studies.

Experimental design.

The rhesus monkey was restrained in a chair for 5 days before inoculation. A single inoculum of Rickettsia rickettsii (10^2 - 10^3) was administered SC (medial aspect of the thigh). Polyethylene catheters were implanted into the femoral artery and vein 6 days after infection. Measurements of body fluid compartments and calculation of F_{cell} ratios were conducted at 8 days. When the monkey was terminal, tissue samples were taken for analysis of water contents and electrolyte distribution analysis.

Body fluid compartment.

Fever and rickettsemia began on day 5 after infection. Although plasma Na^+ and K^+ remained unaltered during RMSF, body fluid retention was observed, as shown by slight increases in total body water as well as plasma, RBC, and blood volumes. Increased F_{cell} ratio values (from 0.88 to 1.04) suggest that vasodilatation and peripheral blood pooling may be present during RMSF.

Tissue water and electrolytes.

Although water and electrolytes in the cardiac muscle (left ventricle) remained relatively constant, gastrocnemius muscle and lung revealed extracellular water accumulation and intracellular dehydration (Table VII). The cause appeared to be from shifts of Na^+ and Cl^- outside of cells. The renal cortex showed decreases of total and intracellular Na^+ contents, which indicated that the mechanism of renal reabsorption may be impaired. The liver showed opposite responses compared to skeletal muscle, i.e., increase in intracellular water and a decrease in extracellular water, with simultaneous reductions of extracellular Na^+ as well as intra- and extracellular K^+ .

TABLE VII: TISSUE WATER AND ELECTROLYTE CHANGES 8 DAYS AFTER INOCULATION OF R. RICKETTSII IN RHECUS MONKEYS (n=2).

TISSUE	GROUP	H_2O (cc/kg FFWT ^a)		ELECTROLYTES (mEq/kg FFWT)					
		T	E _E	T ^b	K ⁺	Cl ⁻	Na ⁺	E _C	K ⁺
Heart	Control	769	262	506	46	94	34	38	0.87
	RMSF	810	202	608	39	86	26	29	0.79
Gastro-nemius muscle	Control	767	123	644	41	96	17	17	0.41
	RMSF	835	316	518	48	80	40	46	1.21
Lung	Control	809	330	479	87	63	44	46	1.10
	RMSF	881	778	102	81	57	90	111	3.19
Liver	Control	796	321	476	40	90	41	45	1.06
	RMSF	793	140	652	40	86	19	20	0.54
Renal cortex	Control	820	306	513	68	73	40	43	1.02
	RMSF	834	361	473	49	80	45	52	1.40

^aFFWT = Fat-free wet tissue.

^bT = mEq total electrolytes/kg FFWT.

^cE = mEq extracellular electrolytes/kg FFWT.

^dI = mEq intracellular electrolytes/kg intracellular H₂O.

^eE = mEq extracellular electrolytes/kg extracellular H₂O.

Presentation:

Liu, C. T., M. J. Griffin, and R. T. Faulkner. Effect of staphylococcal enterotoxin B (SEB) on body fluid compartments in conscious rhesus monkeys. Presented, Fall Meeting, American Physiological Society, San Francisco, CA, 5-10 October 1975. (Physiologist 18:295, 1975)

Publications:

1. Liu, C. T., and G. A. Higbee. 1976. Determination of body surface area in the rhesus monkey. *J. Appl. Physiol.* 40:101-104.
2. Liu, C. T., J. D. Helm, III, and W. R. Beisel. 1976. Cardiovascular and vomiting responses to a lethal intravenous dose of staphyloenterotoxin A in rhesus monkeys. *J. Med. Primatol.* 5: in press.
3. Liu, C. T. 1976. Cardiovascular and renal functions in normal macaques. *Am. J. Vet. Res.* 37: in press.
4. Liu, C. T., M. J. Griffin, and R. T. Faulkner. 1976. Effect of staphylococcal enterotoxin B (SEB) on body fluid compartments in conscious rhesus monkeys. *J. Med. Primatol.* 5: in press.

LITERATURE CITED

1. Finegold, M. J. 1967. Interstitial pulmonary edema. An electron microscopic study of the pathology of staphylococcal enterotoxemia in rhesus monkeys. *Lab. Invest.* 16:912-924.
2. Stiles, J. W., and J. C. Denniston. 1971. Response of the rhesus monkey, *Macaca mulatta*, to continuously infused staphylococcal enterotoxin B. *Lab. Invest.* 25:617-625.
3. Harrell, G. T. and J. K. Aikawa. 1949. Pathogenesis of circulatory failure in Rocky Mountain spotted fever. Alterations in the blood volume and the thiocyanate space at various stages of the disease. *Arch. Intern. Med.* 83:331-347.
4. Harrell, G. T. 1960. Physiologic alterations in patients with spotted fever, p. 23-38. In symposium on the Spotted Fever Group of Rickettsiae (Ed. C. L. Wisseman). *Med. Sci. Pub. No. 7*, Walter Reed Army Institute of Research, U.S. Government Printing Office, Washington, D.C.
5. Sammons, L. S., R. H. Kenyon, G. T. Burger, C. E. Pedersen, and R. O. Spertzel. 1976. Changes in blood serum constituents and hematologic parameters in rhesus monkeys infected with Rocky Mountain spotted fever. *Am. J. Vet. Res.* 37:725-730.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^b	2. DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL
3. DATE PREV SURVEY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCY ^b U	6. WORK SECURITY ^b U	7. REGRADING ^b NA	8. DOD/N INSTRN ^b NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
				10. NO./CODES: ^b PROGRAM ELEMENT 62760A	PROJECT NUMBER 3A762760A834	11. LEVEL OF SUM A. WORK UNIT 111
				12. NO./CODES: ^b c. CONTRIBUTING CARDS 114(e)(f)		
13. TITLE / (Proceed with Security Classification Code) ^a (U) Lysosomal activation in polymorphonuclear neutrophils						
14. SCIENTIFIC AND TECHNOLOGICAL AREA ^a 003500 Clinical medicine; 004900 Defense; 012300 Biochemistry						
15. START DATE 73 09	16. ESTIMATED COMPLETION DATE CONT	17. FUNDING AGENCY DA	18. PERFORMANCE METHOD C. In-house			
19. CONTRACT/GRANT & DATES/EFFECTIVE: b. NUMBER: ^b c. TYPE: d. KIND OF AWARD:	EXPIRATION: NA	20. RESOURCES ESTIMATE FISCAL YEAR 76	21. PROFESSIONAL MAN YRS CURRENT 1.0			
	e. AMOUNT: f. CUM. AMT.	YEAR 77	22. FUNDS (in thousands) 86			
23. RESPONSIBLE DOD ORGANIZATION NAME: ^b USA Medical Research Institute of Infectious Diseases ADDRESS: ^b Fort Detrick, MD 21701	24. PERFORMING ORGANIZATION NAME: ^b Bacteriology Division USAMRIID ADDRESS: ^b Fort Detrick, MD 21701					
25. RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833	26. PRINCIPAL INVESTIGATOR (Provide DEAN if U.S. Academic Institution) NAME: ^b Rausch, P. G. TELEPHONE: 301 663-7341 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: Dangerfield, H. G. NAME:					
27. GENERAL USE Foreign intelligence considered	28. POC:DA					
29. KEYWORDS (Proceed EACH with Security Classification Code) (U) Specific granules; (U) Bacteria; (U) Myeloperoxidase; (U) Leukocytes; (U) Lysosomes; (U) Alkaline phosphatase (AkP); (U) Lysozyme (LZM)						
30. TECHNICAL OBJECTIVE, & APPROACH, & PROGRESS (Provide individual paragraphs identified by number. Proceed each with Security Classification Code) 23 (U) Define the response of lysosomal enzymes in circulating polymorphonuclear (PMN) leukocytes during infection and/or immunization. This work unit represents an investigation aimed at strengthening host abilities to defend against potentially important bacterial BW agents. 24 (U) Prepare specific antisera in goats against AkP and LZM. Use them as reagents for evaluation of changes in rabbits after challenge with bacteria. 25 (U) 75 07 - 76 06 - Continued investigations of rabbit PMN AkP suggest that it exists as 3 isoenzymes, 2 of which can be separated by DEAE chromatography. Enzyme has been isolated by butanol and SDS extraction from glycogen-induced peritoneal exudate cells, peripheral PMN and bone marrow cells. Regardless of source, all have an approximate MW of 500,000 and an isoelectric point of 5.5 - 6.0. Electrophoresis equipment has been ordered to allow rapid purification of enzymes from various sources. LZM has also been extracted from rabbit PMN. While disc electrophoresis indicates the material is pure, goats have failed to produce antibodies against LZM. Electron microscopy of rhesus monkey PMN indicate that 2 morphologically distinct granule populations are present and that myeloperoxidase is confined to a single class of granules.						
Publications: Infect. Immunity 12:687-693, 1975. Blood 46:913-919, 1975.						
Available to contractors upon originator's approval.						

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infections of Military Importance

Work Unit No. 834 01 111: Lysosomal Activation in Polymorphonuclear Neutrophils

Background:

The mechanisms controlling induction of polymorphonuclear neutrophil (PMN) lysosomal enzymes during infection have long been of interest. Numerous studies in animals and man have shown that the mechanisms by which alkaline phosphatase (AkP), acid phosphatase, β -glucuronidase (β -GLUC) and occasionally myeloperoxidase (MPO) are activated are unknown. While glucocorticoids have been shown to be potent AkP activators *in vivo*, other studies have demonstrated that infection appears to activate the enzyme by means of a steroid-independent mechanism.

Data previously reported from this laboratory are in agreement with these findings. We have shown that AkP is undetectable in normal rhesus monkey PMN, but rises to very high levels during infection with Salmonella typhimurium. In another series of experiments, it was found that while glucocorticoids activate serum liver-derived AkP, they had no effect on PMN AkP.

Unfortunately, such studies do not allow conclusions regarding biochemical mechanisms of enzyme induction. An enzyme may be induced by additional synthesis of the active molecule or preexistent enzyme activation through generation of necessary cofactors or by conformational changes in molecular structure. To distinguish these possibilities it is necessary to quantitate intracellular enzyme levels in absolute terms as well as by more conventional kinetic analyses. To this end, AkP was selected as the marker enzyme, and rabbits as the experimental model. The enzyme was purified from peritoneal exudate PMN by means of butanol (BuOH) extraction (BTE), serial $(\text{NH}_4)_2\text{SO}_4$ precipitation, and Sephadex G-200 chromatography. The final enzyme preparation (BTE-AkP) was employed as an immunogen.

Progress:

As reported previously the antiserum to BTE-AkP produced in goats was not monospecific and appeared to precipitate rabbit albumin and 2 minor, non-enzymatic cellular components. An electroimmunoassay with the goat anti-serum was developed according to the methods of Laurell.² While the technique was effective in quantitating standards of purified antigen, its sensitivity

was not sufficient to detect the low levels of enzyme present in unstimulated PMN. Attempts to increase sensitivity with radioautography were similarly unsuccessful.

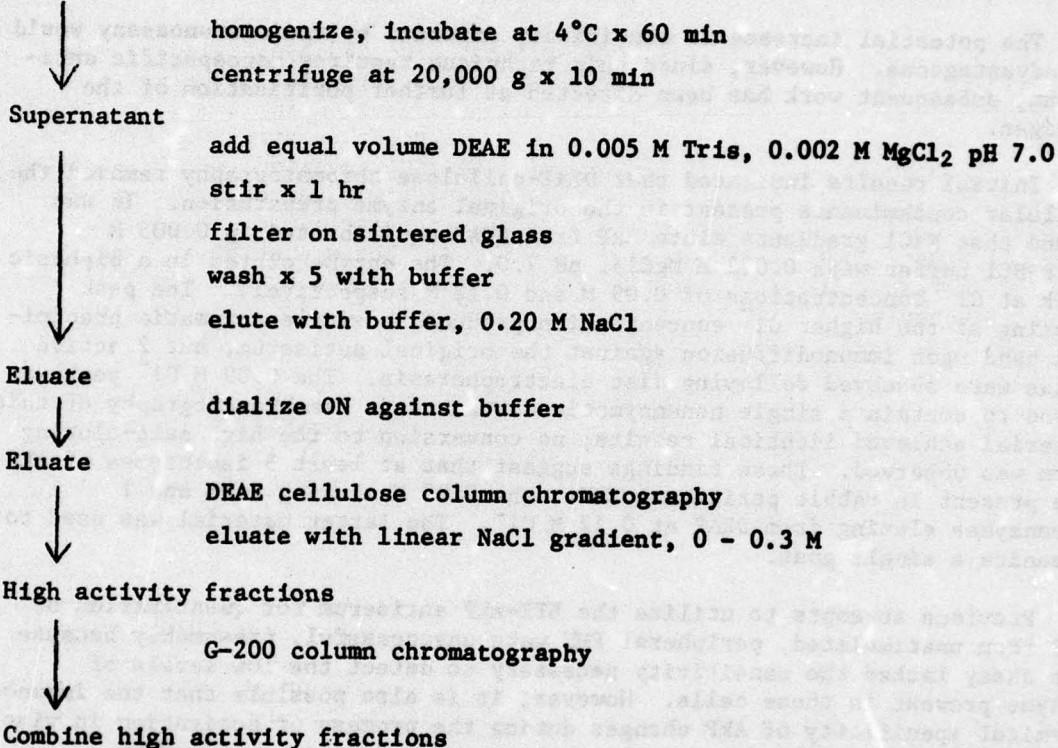
The potential increase in sensitivity afforded by radioimmunoassay would be advantageous. However, since this technique requires monospecific anti-serum, subsequent work has been directed at further purification of the antigen.

Initial results indicated that DEAE-cellulose chromatography removed the cellular contaminants present in the original enzyme preparation. It was found that NaCl gradients elute AkP from DEAE equilibrated in 0.005 M Tris-HCl buffer with 0.002 M MgCl₂, pH 7.0. The enzyme eluted in a biphasic peak at Cl⁻ concentrations of 0.09 M and 0.12 M respectively. The peak eluting at the higher Cl⁻ concentration produced a single enzymatic precipitin band upon immunodiffusion against the original antiserum, but 2 active bands were observed following disc electrophoresis. The 0.09 M Cl⁻ peak was found to contain a single nonenzymatic contaminant. Rechromatography of this material achieved identical results; no conversion to the high salt-eluting form was observed. These findings suggest that at least 3 isoenzymes of AkP are present in rabbit peritoneal PMN: the 0.09 M eluting form and 2 isoenzymes eluting from DEAE at 0.12 M Cl⁻. The latter material was used to immunize a single goat.

Previous attempts to utilize the BTE-AkP antiserum for quantitation of AkP from unstimulated, peripheral PMN were unsuccessful, presumably because the assay lacked the sensitivity necessary to detect the low levels of enzyme present in these cells. However, it is also possible that the immunochemical specificity of AkP changes during the process of activation *in vivo*, or as a result of the organic solvent extraction during purification. To distinguish these possibilities, experiments were undertaken utilizing sodium dodecyl sulfate (SDS) extraction in place of BuOH. Initial trials attempted to isolate the PMN granules from peritoneal exudate cells by homogenization in 0.34 M sucrose, differential centrifugation and SDS extraction of the granule fraction. This approach was abandoned when it was found that the agglutinated cells produced inadequate yields of granules for enzyme extraction. In contrast, homogenization and SDS solubilization of whole cell preparations produced excellent yields of AkP (10-fold greater than from isolated granules and 4-fold greater than BuOH extraction), but simultaneous DNA solubilization resulted in the formation of a thick gel which rendered subsequent manipulations impossible. Addition of DNase and incubation of the mixture at 37°C for 4 hr resulted in decreased viscosity but a major portion of the AkP was also inactivated. Subsequently, it was found that the AkP could be effectively isolated by DEAE batch absorption; this method (Table I) was used in subsequent experiments (SDS-AkP).

TABLE I. AkP PURIFICATION: SDS METHOD

10% WBC (W/V) in 0.005 M Tris, 0.002 M MgCl₂, 1% SDS pH 7.0



To determine characteristics of BTE-AkP and SDS-AkP, the MW of all preparations were estimated by Sephadex G-200 chromatography. Enzymes prepared by both methods were found to have MW of 450,000 - 500,000 daltons. Similarly, AkP prepared from unstimulated rabbit peripheral PMN and from rabbit bone marrow was found to have identical molecular weights.

It should be noted that we previously reported¹ the MW of BTE-AkP to be 190,000. Although the reasons for this conflict are presently unknown, the original estimate was made using Bio Gel A 1.5 chromatography with 0.1% Triton X 100 in the buffer. Further investigation is in progress.

SDS-AkP reacted with BTE-AkP antiserum in a manner identical to that of BTE-AkP; immunologic lines of identity could be seen between both forms of the enzyme.

Since the 2 purification methods did not appreciably affect either MW or immunochemical identity of AkP, studies were undertaken of AkP obtained from unstimulated peripheral PMN. However, these efforts were frustrated by the low activity of the enzyme in unstimulated PMN (~ 200 nM/10⁷ cells/min) as compared to that present in exudate cells.

In the first experiment, dextran sedimentation and hypotonic lysis were employed to isolate WBC from 350 ml of anticoagulated blood obtained from 4 rabbits. The resultant pellet (0.61 gm), homogenized in 1% SDS, released 402 nM/ml/min of AkP activity. Seventy-five percent recovery was achieved from DEAE batch absorption. This material was then applied to DEAE and G-200 chromatographic columns. The final product was tested by immuno-diffusion and immunoelectrophoresis. Enzyme activity could not be detected by histochemical techniques applied to the gels, but a precipitin band, similar to that seen with peritoneal BTE-AkP, was demonstrated with coomassie blue. Nonetheless, final quantities obtained were insufficient to serve as an immunogen.

In an attempt to obtain a greater yield of unstimulated cells, femoral bone marrow (3.3 gm) was obtained from 5 rabbits and processed as before. In this case, the initial homogenate contained 578 nM/ml and the yield from DEAE absorption was 70%. Insufficient quantities of purified enzyme were obtained for detailed studies.

Because of difficulties in obtaining sufficient quantities of unstimulated enzyme, electrofocusing studies were undertaken in an attempt to increase yield. Initial experiments using BTE-AkP in acrylamide gels indicated a pI of $\sim 5.5 - 6.0$. Accordingly, BTE-AkP was applied to a preparative electro-focusing column. The results indicated that while the enzyme obtained had a high degree of purity, precipitation occurred at the isoelectric point and the yield was accordingly reduced.

Electrofocusing in granulated gels (Sephadex G-75) has been said to avoid these problems, and preliminary experiments were performed utilizing an electrophoretic apparatus fabricated by the engineering department. Fair separation was achieved with a high yield; however, because this apparatus only has a 10-cm running distance, the separation was incomplete. Accordingly, the LKB Multipore electrofocusing apparatus has been ordered and should allow complete separation of AkP in high yield, with little inactivation. It is anticipated that this approach will allow direct comparisons of purified AkP from both activated and unstimulated cells.

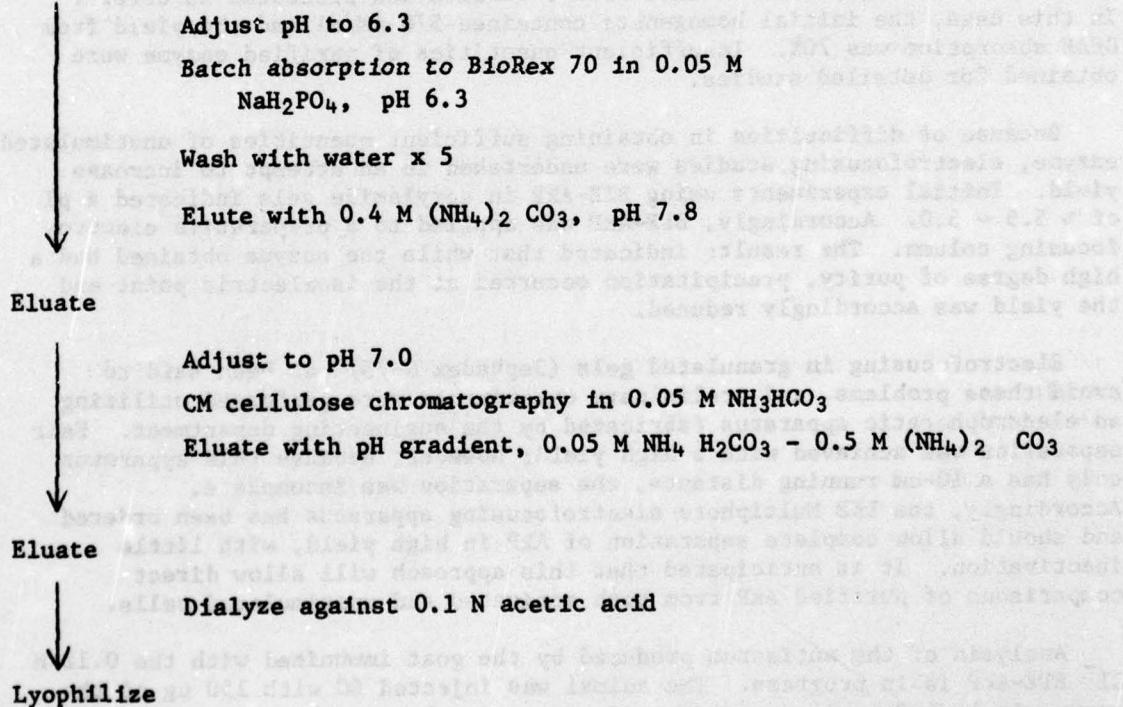
Analysis of the antiserum produced by the goat immunized with the 0.12 M Cl⁻ BTE-AkP is in progress. The animal was injected SC with 150 μ g of the enzyme in 1 ml Freund's complete adjuvant and subsequently injected at 3-wk intervals with a similar amount of protein in Freund's incomplete adjuvant. Precipitable antibody appeared by day 22; titers increased following subsequent boosters. The IgG fraction has been isolated by the method of Houston et al.³ and is presently being evaluated for specificity.

In other species, AkP is a Zn^{++} metalloenzyme and Mg^{++} is necessary for full expression of activity. To determine if these metals are structurally contained in rabbit AkP, the 0.12 M Cl^- BTE-AkP was exhaustively dialyzed against buffer free of Zn^{++} and Mg^{++} ; Zn and Mg concentrations were then determined by atomic absorption spectrophotometry. Assuming a MW of 500,000 for AkP, the results indicated that there were 2.2 and 1.6 atoms of Zn and Mg respectively per molecule of AkP.

In a search for other granule markers in rabbit PMN, studies of basic proteins (contained within azurophilic granules) and lysozyme (LZM -- a marker for both azurophilic and specific granules) were undertaken. Since all are highly cationic, they were extracted from rabbit peritoneal cells by 0.2 N H_2SO_4 ; LZM was purified by the method of Canfield et al.⁴ (Table II).

TABLE II. LZM PURIFICATION.

0.2 N H_2SO_4 extract



The procedure resulted in a 20-fold increase in specific activity of LZM with a 60% yield. The final product demonstrated a single protein band upon disc electrophoresis at pH 4.5; 15 mg were obtained. One mg in Freund's complete adjuvant was used to immunize a goat; booster injections in incomplete

adjuvant were given at 2-wk intervals. Despite a total of 3 injections, anti-LZM antibodies are not detectable by immunodiffusion. This may well be due to documented genetic similarities of LZM between various species.

In continuing investigations of the biochemical and morphological characteristics of rhesus monkey PMN, we sought to identify the granule types present in these cells and to localize specific enzymes within granules by cytochemical techniques. Electron micrography of normal rhesus PMN was performed by Dr. White of the Pathology Division (Work Unit 834 01 406). Two morphologically distinct granules were identified, a large, spherical, electron-dense particle and a smaller, less dense, rod-shaped granule. While these correspond to morphologic findings in human and rabbit PMN, specific cytochemical stains are required to demonstrate enzymatic partition between the 2 classes. MPO was stained⁵ and was shown to be entirely contained within the former granule type. We were able to confirm fusion of these particles with phagocytic vacuoles and discharge of MPO into the phagolysosome. Present efforts are directed at the demonstration of AkP in PMN isolated from monkeys infected with S. typhimurium. Unfortunately, technical difficulties have been encountered and the enzyme has not been demonstrated convincingly. Average processing time continues to be 5-6 wk for individual samples.

We previously reported very preliminary experiments which suggested that LEM was capable of inducing high levels of rabbit PMN AkP *in vitro*. However, our continuing inability to obtain additional samples of LEM have prevented confirmation of this intriguing finding.

Publications:

1. Rausch, P. G., and P. G. Canonico. 1975. Characterization of monkey peripheral neutrophil granules during infection. *Infect. Immun.* 12: 687-693.
2. Rausch, P. G., and T. G. Moore. 1975. Granule enzymes of polymorphonuclear neutrophils: a phylogenetic comparison. *Blood* 46:913-919.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1975. Annual Progress Report, FY 1975. p. 79-86. Fort Detrick, Frederick, MD.
2. Laurell, C.-B. 1972. Electroimmuno assay. *Scand. J. Clin. Lab. Invest.* 29(Suppl. 124):21-37.

3. Houston, W. E., C. E. Pedersen, Jr., F. E. Cole, Jr., and R. O. Spertzel. 1974. Effects of antigen-antibody complexes on the primary immune response in rhesus monkeys. *Infect. Immun.* 10:437-442.
4. Canfield, R. E., J. C. Collins, and J. H. Sobel. 1974. Human leukemia lysozyme, p. 63-70. *In Lysozyme*. (ed. E. F. Osserman, R. E. Canfield, and S. Beychok), Academic Press, New York.
5. Graham, Jr., R. C., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14:291-302.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^b	2. DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL
3. DATE PREV SURRY 76 01 19	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY ^b U	6. WORK SECURITY ^b U	DA OP6425	76 07 01	DD-DR&E(AR)636
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10. NO./CODES: ^b a. PRIMARY 62760A	PROGRAM ELEMENT PROJECT NUMBER 3A762760A834	TASK AREA NUMBER 01	WORK UNIT NUMBER 112			
b. CONTRIBUTING	c. EQUIPMENT CARDS 114(e)(f)					
11. TITLE (Pecode with Security Classification Code) (U) Role of the kallikrein-kinin system in infectious diseases						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^b 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry						
13. START DATE 75 12	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house			
17. CONTRACT/GRAANT a. DATES/EFFECTIVE: b. NUMBER: ^b NA c. TYPE: d. KIND OF AWARD:		EXPIRATION:	18. RESOURCES ESTIMATE FISCAL YEAR PRECEDING 76	19. PROFESSIONAL MAN YRS CURRENT 0.5	20. FUNDS (In thousands) 66.6	
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19. RESPONSIBLE DOD ORGANIZATION NAME: USA Medical Research Institute of Infectious Diseases. ADDRESS: Fort Detrick, MD 21701		20. PERFORMING ORGANIZATION NAME: Bacteriology Division USAMRIID ADDRESS: Fort Detrick, MD 21701				
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Junior Dean // U.S. Academic Institution) NAME: Yamada, T. TELEPHONE: 301 663-7341 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: Pettit, G. W. NAME: Wing, D. A. POC:DA				
21. GENERAL USE Foreign intelligence considered						
22. TECHNICAL OBJECTIVE, ^b 24. APPROACH, 25. PROGRESS (Pecode individual paragraphs identified by number. Pecode rest of each with Security Classification Code.) 23 (U) Investigate the role of the kinin system and myocardial depressant factor in pathogenesis of sepsis and shock. This will help to elucidate the pathophysiology of some of the most severe complications associated with infections of unique military significance, i.e. those which pose a potential BW threat. 24 (U) Use various animal models to study the sequence of events in the pathogenesis of sepsis and shock. 25 (U) 76 01 - 76 06 - The kinins and myocardial depressant factor appear to play important roles in the pathophysiology of shock and other complications of sepsis by virtue of their profound cardiovascular actions. Assays were established to quantitate their activities in plasma. Animal models for sepsis and disseminated intravascular coagulation were developed using both Streptococcus pneumoniae and Salmonella typhimurium as infecting organisms; studies in these models indicated that kinin system activation, along with activation of coagulation and fibrinolysis, was part of the normal host response to infection, regardless of severity of illness. Activation of kinins was also observed in rickettsial and viral infections. Studies in monkeys subjected to staphylococcal enterotoxin B-induced shock indicated that while early fall in blood pressure was related to kinin system activation, terminal hypotension was related to production of myocardial depressant factor.						
Available to contractors upon originator's approval.						

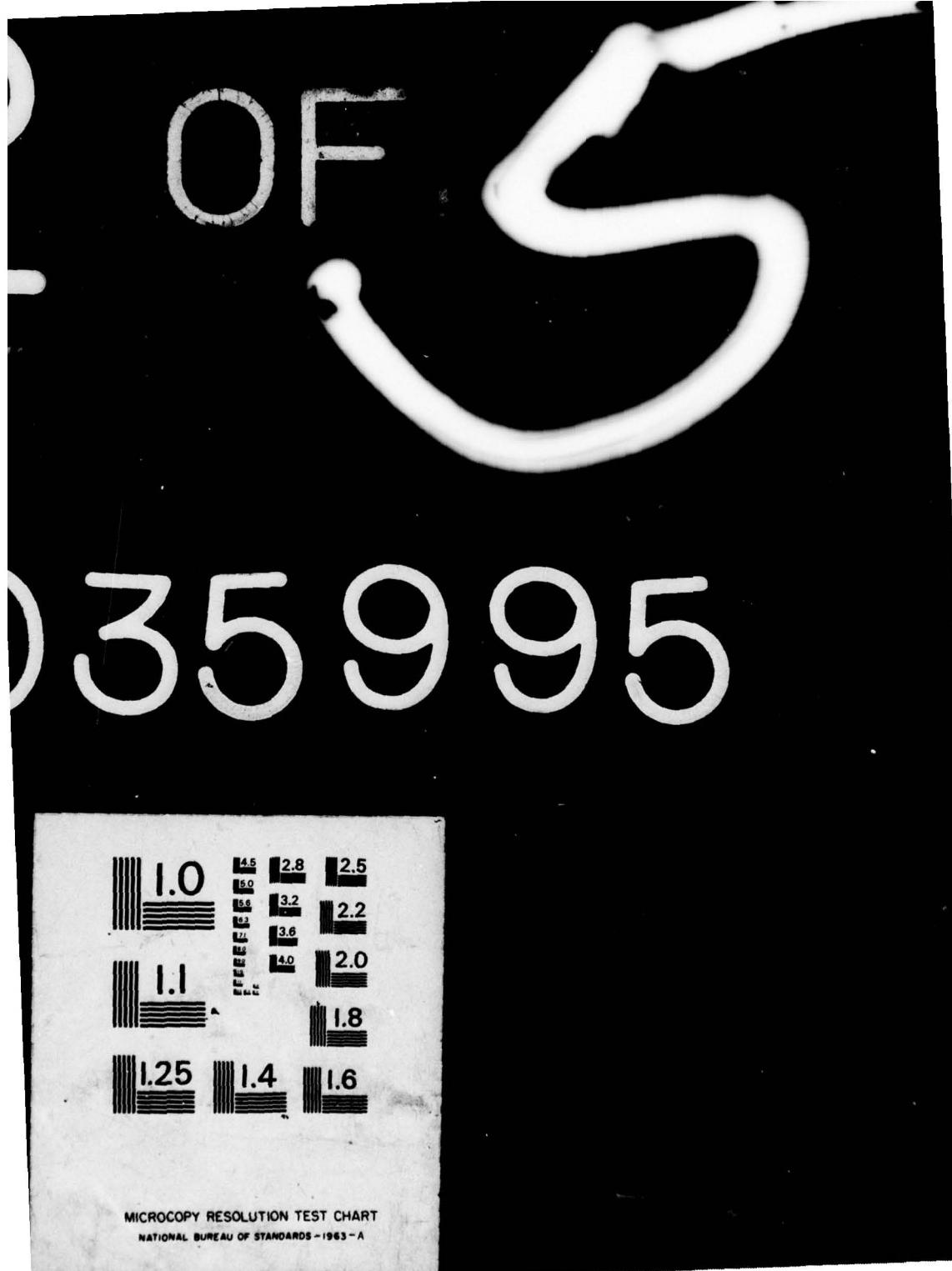
AD-A035 995 ARMY MEDICAL RESEARCH INST OF INFECTIOUS DISEASES FR--ETC F/G 6/5
ANNUAL PROGRESS REPORT - FY 1976.(U)
JUL 76 F B ABELES, A O ANDERSON, J B ARENSMAN

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BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 112: The Role of The Kallikrein-Kinin System In Infectious Diseases

Background:

The kinins are a group of polypeptides known for (1) their ability to mimic inflammation by causing vasodilation, increased vascular permeability, chemotaxis, and pain, and (2) their profound cardiovascular effects resulting from peripheral vasodilation as well as direct catecholamine release. The kinin system interacts closely with the complement, coagulation and fibrinolytic systems to mediate host response to injury such as infection. Because of these actions, the kinin system has been implicated in the pathophysiology of septic shock. Nies and his associates¹ injected endotoxin into unanesthetized rhesus monkeys and observed a close relationship between kinin system activation and the early reversible phase of endotoxin shock. No correlation was observed, however, between kinins and the terminal irreversible shock associated with endotoxemia. Other investigators have attempted to study the mechanisms involved in the terminal irreversible phase of septic shock by implicating various neurohumoral transmitters and vasoactive substances such as norepinephrine, acetylcholine, histamine, serotonin, vasopressin, ferritin, and angiotensin II, but none of these substances have been found during septic shock in concentrations great enough to cause definite toxicity. However, in this regard, Lefer and coworkers²⁻⁴ detected the presence of a toxic substance, subsequently termed myocardial depressant factor (MDF), in plasma during splanchnic vessel ischemia, such as might occur in septic shock. IV injection of MDF into intact cats, employing concentrations similar to those observed during splanchnic artery occlusion, resulted in terminal irreversible shock. Since the kinins and MDF may play important roles in the development of septic shock, investigation of their activities during the various phases of shock associated with sepsis was undertaken in order to elucidate the complicated pathophysiologic mechanisms involved in this difficult clinical problem.

Another clinically troublesome complication of sepsis is disseminated intravascular coagulation (DIC), a disorder thought to be caused by consumption of coagulation factors. Although the primary clinical manifestation of DIC is hemorrhage, hypotension in DIC is rarely attributable to uncontrolled bleeding. The known cardiovascular effects of kinins, coupled to their close association with coagulation and fibrinolysis, suggest that

they may be important in the sequence of events leading to DIC. Thus, investigations of the various roles of the kinin, coagulation, and fibrinolytic systems in DIC were initiated in order to define better the clinical entity, as well as to gain insight into its pathogenesis.

Progress:

Assay methods for kinin system components and MDF. Initial efforts were directed toward developing assay methods for kinin system components and MDF. Activation of the kinin system was measured by quantitating changes in plasma kallikrein, prekallikrein, and kininogen. Kallikrein was assayed by measuring spontaneous 1-tosylarginine methylesterase (TAMe) activity of plasma. Prekallikrein was converted to kallikrein by kaolin-activation and TAMe activity was measured; the difference between kaolin-activated TAMe activity and spontaneous TAMe activity was equivalent to prekallikrein activity. Kininogen was measured by bioassay on guinea pig ileum of kinin generated from plasma after treatment with human urinary kallikrein (supplied by J. C. Pierce, NIH). Normal values obtained for human and rhesus monkey kinin components are presented in Table I. The values obtained for human kallikrein, prekallikrein, and kininogen are consistent with generally-accepted normal values. Normal values for kinin system components in monkeys have not been reported previously.

TABLE I. NORMAL VALUES FOR HUMAN AND RHESUS MONKEY KININ COMPONENTS.

	MEAN VALUE \pm SD (n)	
	Human	Rhesus monkey
Kallikrein ($\mu\text{mol}/\text{ml}/\text{hr}$)	3.4 \pm 0.9 (n = 6)	11.0 \pm 3.6 (n = 32)
Prekallikrein ($\mu\text{mol}/\text{ml}/\text{hr}$)	103.4 \pm 8.5 (n = 6)	145.2 \pm 29.8 (n = 32)
Kininogen ($\mu\text{g}/\text{ml}$)	1.09 \pm 0.23 (n = 3)	0.32 \pm 0.03 (n = 7)

Bioassayable MDF was measured in the following fashion: plasma ultrafiltrates (passed through a 20,000 MW molecular filter) were chromatographed on a 90 x 1.5 cm polyacrylamide gel column (Biogel P-2, 200-400 mesh) and samples collected at elution volumes of 85-105 ml were applied to an isolated rabbit papillary muscle preparation. One MDF unit was defined as that amount that caused a 1% depression in developed tension in the papillary muscle. The validity of the bioassay in the rabbit model was tested by measuring MDF in 3 normal rabbits and 3 rabbits exposed to hemorrhagic shock. The mean MDF for control rabbits was 6.8 ± 3.9 U while the mean for the

shocked rabbits was 18.1 ± 2.3 , a difference that was significant ($P < 0.05$). In addition, preshock and shock MDF values were obtained for a single rabbit and these values were 0 and 19 U respectively.

MDF was also measured by paper chromatography using a newly-developed modification of a method described by Barenholz et al.⁴ Heparinized plasma samples were deproteinized by trichloroacetic acid precipitation and extracted with ether. Processed samples were applied to Whatman's No. 3 MM paper and the paper was saturated with solvent (n-butanol, glacial acetic acid, and double-distilled water) applied via descending flow for 14-18 hr. The papers were then sprayed with 0.3% ninhydrin in ethanol and developed at 90 C for 10 min. The serine standard spot and the spots containing all bioassayable MDF (Spot G, $R_s = 1.6 - 1.8$) were eluted from the paper using 1% NaHCO₃ and absorbance of the eluate was measured at 570 nm. One MDF chromatographic unit was equal to A_{570} of 1.3 nmol of serine. There was excellent correlation ($r = 0.99$) between serial dilutions of a single plasma sample and A_{570} of Spot G eluate of those dilutions, as well as excellent correlation ($r = 0.97$) between bioassay and paper chromatography determinations for 16 plasma samples. Duplicate variability for the 16 samples did not exceed 15% and averaged 2.6%. Development of this paper chromatographic assay has not only facilitated the measurement of MDF, but has also provided the capability of serially measuring MDF in an animal over a period of time using only minimal amounts of plasma (<100 μ l/assay).

Animal models for disseminated intravascular coagulation. In collaboration with MAJ H. B. Hawley (Bacteriology Division, Work Unit 834 03 103) and CPT D. A. Wing (Pathology Division, Work Unit 834 01 007), animal models for sepsis and DIC were developed using both Streptococcus pneumoniae and Salmonella typhimurium as infecting organisms.

Ten rhesus monkeys (Macaca mulatta) were utilized for the S. pneumoniae type I (ATCC strain 6301) model. The monkeys were allocated to 3 groups. In group A, consisting of 4 normal monkeys, 3 were infected with 1×10^8 S. pneumoniae IV and 1 was injected with normal saline. In group B, containing 3 asplenic monkeys, 2 were infected with 1×10^3 S. pneumoniae, IV and the 3rd was injected with normal saline. In group C, containing 3 asplenic monkeys, 2 were infected with 1×10^6 S. pneumoniae IV and 1 was injected with normal saline. The monkeys were followed with serial measurements of various physical and laboratory parameters beginning 4 days prior to infection and continuing until the 10th day postchallenge. Results are depicted in Tables II and III. The infected monkeys in groups A and C were quite ill with markedly diminished activity and food intake. These monkeys showed reductions in platelet counts (except monkey 10), increases in FSP, and activation of their kinin systems, as demonstrated by decreasing pre-kallikrein and increasing kallikrein, all evidence of DIC. While group B infected monkeys were not clinically ill, they too showed evidence of DIC in that their kinin systems appeared to be activated (Table II).

TABLE II. SUMMARY OF PLATELET COUNT, FIBRIN SPLIT PRODUCTS, PREKALLIKREIN, AND KALLIKREIN OBSERVED IN RHESUS MONKEYS INFECTED WITH S. PNEUMONIAE TYPE I.

MONKEY Group	No.	CHALLENGE	PLATELET COUNT (cells/mm ³)	MINIMUM FSP (μ g/ml)	MAXIMUM FSP (μ g/ml)	KALLIKREIN		PREKALLIKREIN	
						Control ^a	Maximum	Control ^a	Minimum
A (normal)	1	Saline	225,000	< 5.0	9	12	120	110	110
	2	1×10^8	89,000	20.0	13	19	116	76	76
	3	1×10^8	147,000	20.0	9	16	116	64	64
	4	1×10^8	73,000	30.0	10	14	122	75	75
	5	Saline	376,000	ND	13	15	110	102	102
B (asplenic)	6	1×10^3	298,000	ND	6	9	113	88	88
	7	1×10^3	234,000	ND	8	17	121	84	84
	8	Saline	136,000	< 5.0	12	15	116	108	108
C (asplenic)	9	1×10^6	24,000	9	9	16	100	33	33
	10	1×10^6	286,000	12	14	23	122	80	80

^a Pre-infection values.

^b ND, not determined.

TABLE III. ACUTE AND CONVALESCENT VALUES FOR PLATELET COUNT, FIBRIN SPLIT PRODUCTS, PLASMA KALLIKREIN AND PREKALLIKREIN, AND HAGEMAN FACTOR IN A PATIENT WITH RMSF.

	CONTROLS (n = 6) (mean value \pm SD)	PATIENT'S VALUES	
		Acute	Convalescent
Platelets (cells/mm ³)	200,000 \pm 80,000	50,000	280,000
FSP (μ g/ml)	0	25	0
Kallikrein (μ mol/ml/hr)	3.4 \pm 0.9	17.2	4.4
Prekallikrein (μ mol/ml/hr)	103.4 \pm 8.5	59.2	101.0
Hageman factor (% of normal)	102 \pm 20	70	ND ^a

^a ND = not determined.

Attempts to activate both the kinin and complement systems in vitro with pneumococcal capsular polysaccharide (PCP) were unsuccessful. Complement components C3, C4 and C5, measured by radial immunodiffusion, were increased in the infected monkeys in all 3 groups and there was no evidence of complement consumption (data presented in Work Unit 834 03 103). Significant type-specific antibody production in the infected monkeys did not occur until at least day 3 postchallenge and there appeared to be a relationship between the appearance of antibody and the disappearance of PCP. Asplenic infected monkeys showed little or no antibody response. The implications and conclusions of this study were as follows: (1) a working model for DIC in pneumococcal sepsis was established in rhesus monkeys, (2) severity of clinical illness in infection depended upon infecting dose of organisms as well as upon host resistance factors (i.e., the presence of a spleen), (3) neither complement consumption, antigen-antibody complex formation, nor PCP activation of Hageman factor appeared to play a role in the initiation of DIC, (4) activation of the kinin, coagulation, and fibrinolytic systems, as reflected in laboratory changes consistent with DIC, represented a normal response of the host to infection even in the absence of clinical signs of illness.

S. typhimurium (USAMRIID strain)-induced DIC model was developed employing cynomolgus and rhesus monkeys. In the initial studies cynomolgus monkeys were injected IV with 1×10^9 organisms of S. typhimurium; severe clinical illness resulted and was characterized by prostration, petechial rash and ultimately death. Activation of fibrinolytic (FSP > 20 $\mu\text{g}/\text{ml}$), coagulation (platelet count < 25,000/ mm^3), and kinin systems (prekallikrein reduction > 50%) was observed in all infected monkeys. Bioassayable MDF was markedly increased (> 50 U) in the one infected, terminally ill monkey in which it was measured. Although the DIC model in cynomolgus monkeys was adequate for study, it was felt that a similar model in rhesus monkeys would be more practical because the larger monkeys' greater blood volume would allow more sampling of blood for laboratory studies without producing significant hemodynamic changes. IV injection of 1×10^9 S. typhimurium into 11 rhesus monkeys also produced severe clinical illness with prostration and petechial rash but, in contrast to cynomolgus monkeys, all animals recovered completely. Again, activation of fibrinolytic (FSP > 20 $\mu\text{g}/\text{ml}$), coagulation (platelet count < 100,000/ mm^3), and kinin systems (prekallikrein reduction > 30%) was observed in all infected monkeys. Studies measuring fibrinogen turnover in these animals are in progress to characterize further the exact nature of the observed changes in DIC. In addition, purification and isolation of both plasminogen and kininogen from monkeys plasma are currently being attempted in order to conduct turnover studies of these plasma proteins during DIC.

Kinins in rickettsial and viral diseases. A laboratory worker at USAMRIID was accidentally infected with Rickettsia rickettsii and developed classical RMSF with high fever (104.6 F) and constitutional symptoms such as headache, malaise, and myalgia, and petechial rash. In addition, the patient manifested laboratory evidence of DIC: elevated FSP, thrombocytopenia, and kinin system activation (Table III). No evidence of complement consumption was obtained. Evidence for consumption of Hageman factor suggested that the pathophysiology of DIC in this patient involved direct activation of Hageman factor with subsequent activation of coagulation, fibrinolysis, and kinins. The mechanism whereby Hageman factor was activated was unclear but possibilities included (1) platelet aggregation, (2) direct contact with rickettsial organisms, or (3) contact with exposed subendothelial collagen in vessels damaged by either platelet aggregation, direct infection, or antigen-antibody complexes. Complement, however, appeared to play no role in producing DIC in this patient. Since the kinins are known to cause peripheral vasodilation, increased vascular permeability, and leukocyte chemotaxis, their activation in this patient suggested a possible role for the kinins in producing the vascular lesions of RMSF.

Preliminary studies in 4 rhesus monkeys infected with 17-D Yellow fever virus indicated that the kinin system is also activated during viral infections. By day 6 of infection, prekallikrein was decreased and kallikrein was increased in all monkeys. The average decrease in prekallikrein was $20\% \pm 5\%$ and increase in kallikrein was $38\% \pm 21\%$.

Kinins and MDF in SEB-induced shock. MDF was measured in 2 groups of New Zealand white rabbits injected with 1 mg/kg IV of SEB. In the 1st group consisting of 3 rabbits and 4 controls, plasma MDF was measured by bioassay. The SEB-injected rabbits had MDF levels of 44.3 ± 4.8 U whereas MDF levels in the control rabbits were 20.5 ± 1.9 U. After developing the capability to measure MDF by paper chromatography, the experiment was repeated with a 2nd group of rabbits consisting of 4 SEB-injected rabbits and 5 controls. The results were remarkably similar to those of the 1st experiment. Plasma MDF by paper chromatography in the SEB-injected rabbits (1.41 ± 0.63 paper chromatography units) was, again, approximately twice the level observed in control rabbits (0.76 ± 0.06).

Having established the increase of MDF in SEB shock in the rabbit, as well as the validity of the paper chromatographic assay, we initiated studies into the nature of SEB-induced shock in the rhesus monkey. An attempt was made to correlate the cardiovascular events that occur during SEB shock with changes in various vasoactive plasma peptides. SEB (1 mg/kg) was injected IV into 3 rhesus monkeys and the following parameters were measured: central venous pressure (CVP), mean arterial blood pressure (MABP), heart rate (HR), and skin temperature (ST). Plasma samples were withdrawn sequentially for quantitation of platelets, hematocrit, FSP, MDF, kallikrein, prekallikrein, endotoxin, and total hemolytic complement (CH₅₀). Preliminary results obtained for one representative monkey are depicted in Table IV. In this animal, the gradual early decline in MABP was paralleled by progressive decline in plasma pre-kallikrein. This observation suggested a relationship between kinin system activation and the early phase of SEB-induced hypotension, a finding consistent with observations made in endotoxin-induced hypotension as mentioned above.¹ Increase in MDF, however, was not detected until just before death when a precipitous drop in MABP was observed. The data suggested that terminal hypotension in SEB-induced shock in the rhesus monkey correlated with MDF production, although the earlier decline in MABP was related to other factors such as kinin system activation. Laboratory values consistent with DIC were also observed in SEB-injected monkeys as indicated by decreasing platelets and increasing FSP in addition to kinin activation. Data obtained previously in rabbits suggested that death due to SEB-induced shock may be mediated by endogenous endotoxin release.⁵ Measurements of endotoxin by Limulus lysate assay in the SEB-injected monkeys are currently in progress; the results may be helpful in elucidating whether SEB induces shock primarily with secondary endotoxin release, or secondarily through the action of endotoxin.

TABLE IV. SEQUENTIALLY MEASURED PHYSIOLOGIC PARAMETERS, HEMATOCRIT, PLATELETS, FSP, PLASMA KALLIKREIN AND PREKALLIKREIN, MDF, AND CH₅₀ IN MONKEY X-870 INJECTED IV AT 0 HR WITH 1 mg/kg SEB.

PARAMETER	VALUE BY HOURS							
	-1	0	1	3	6	8	11	Preterm. ^a
CVP (cm H ₂ O)	1.5	2.5	2.5	4.0	4.5	3.0	0	0
MABP (mm Hg)	90	85	75	60	65	50	40	
HR (beats/min)	170	170	195	195	195	190	175	
ST (°C)	35	35	35	34.5	34.3	33	30.5	
Hematocrit (%)	48	52	52	57	57	58	63	
Platelets (no. x 10 ⁵ /mm ³)	2.34	2.48	2.15	1.88	1.98	1.84	1.67	
FSP (μg/ml)	0	0	40	20	15	15	15	
Kallikrein (μmol/ml/hr)	11.9	10.9	10.5	11.1	11.1	9.0	8.9	
Prekallikrein (μmol/ml/hr)	185.3	184.4	181.0	159.2	160.0	133.5	133.4	
MDF ^b (p.c. U)	0.125	0.138	0.162	0.184	ND ^c	0.109	0.143	0.382
CH (U)	36	37	32	34	34	31	32	

^a Preterminal.

^b MDF measured in paper chromatography units.

^c ND, not determined

Presentation:

Yamada, T. The kallikrein-kinin system and its role in infectious diseases. Staff Conference, USARIEM, Natick, MA, October 1975.

Publications:

None

LITERATURE CITED

1. Nies, A. S., R. P. Forsyth, H. E. Williams, and K. L. Melon. 1968. Contribution of kinins to endotoxin shock in unanesthetized rhesus monkeys. Circ. Res. 22:155-164.
2. Lefer, A. M., and J. A. Spath, Jr. 1974. Pancreatic hypoperfusion and the production of a myocardial depressant factor in hemorrhagic shock. Ann Surg. 179:868-876.
3. Glenn, T. M., and A. M. Lefer. 1971. Significance of splanchnic proteases in the production of a toxic factor in hemorrhagic shock. Circ. Res. 29:338-349.
4. Barenholz, Y., J. N. Leffler, and A. M. Lefer. 1973. Detection by chemical methods of a myocardial depressant factor in plasma of animals in circulatory shock. Isr. J. Med. Sci. 9:640-647.
5. Pettit, G. W., M. R. Elwell, and P. B. Jahrling. Endotoxemia in rabbits after injection of staphylococcal enterotoxin B. J. Infect. Dis. (submitted for publication).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL
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76 04 05	D. CHANGE	U	U	NA	NL	7. REGADING ^e 8. DISSE'N INSTR'N 9. SPECIFIC DATA-CONTRACTOR ACCESS 10. YES <input checked="" type="checkbox"/> NO <input type="checkbox"/>
10. NO./CODES: ^f	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER	
a. PRIMARY	62760A	3A762760A834		01	300	
b. CONTRIBUTING						
c. EQUIPMENT	CARDS 114(e)(f)					
11. TITLE (Pencils with Security Classification Code) (U) Investigation of the vascular lesions induced by tick-borne rickettsiae						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^g 003500 Clinical medicine; 004900 Defense; 002600 Biology (Pathology)						
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23. PERFORMING ORGANIZATION						
NAME: Pathology Division USAMRIID ADDRESS: Fort Detrick, MD 21701						
PRINCIPAL INVESTIGATOR (Pencils NAME if U.S. Academic Institution)						
NAME: Hall, W. C. TELEPHONE: 301 663-7211 SOCIAL SECURITY ACCOUNT NUMBER:						
ASSOCIATE INVESTIGATORS						
NAME: POC:DA						
24. GENERAL USE Foreign intelligence considered						
(U) Tick-borne rickettsioses; (U) Vascular permeability; (U) Laboratory animals; (U) Pathogenesis; (U) Kinins; (U) Immunity; (U) Military medicine; BW defense						
25. TECHNICAL OBJECTIVE, 26. APPROACH, 28. PROGRESS (Pencils individual paragraphs identified by number. Pencils last of each with Security Classification Code.)						
23 (U) Investigate the pathogenesis of the vascular lesions induced by dangerous nonindigenous rickettsiae. Knowledge of the pathogenesis of such lesions is essential to the understanding of the pathophysiology and planning of rational therapy for the soldier infected by any one of a number of these potential BW agents. Such adjunctive therapy, to be utilized along with antibiotics in treating infected soldiers, will reduce hospitalization time and enable the individual to return to a duty status more rapidly.						
24 (U) Using an appropriate guinea pig model system, study morphologic sequence and location of vascular changes and the influence of immune processes, kinins, etc. on them.						
25 (U) 76 04 - 76 06 - Previous studies under this work unit were concluded by Major J. B. Moe. In his studies, morphologic lesions of highly virulent spotted fever rickettsiae and vascular permeability were temporally correlated with the presence of detectable levels of fibrin split products in blood, and reduction of platelet numbers and rickettsiae in circulating blood. Vascular alteration was not prevented by inactivation of terminal complement components or anticoagulant therapy with heparin.						
This work unit was recently reactivated to investigate further the vascular lesions of life-threatening rickettsial infection. Studies are now in preliminary stages.						
Publication: Vet. Pathol. 13:69-77, 1976.						
Available to contractors upon contract's approval.						
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BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 300: Investigation of the Vascular Lesions Induced by Tick-borne Rickettsiae

Background and Summary:

Previous studies under this work unit were concluded by MAJ J. B. Moe. In his studies, morphologic lesions of Rocky Mountain spotted fever and vascular permeability were temporally correlated with the presence of detectable levels of fibrin split products in blood, and reduction of platelet numbers and rickettsiae in circulating blood. Vascular alteration was not prevented by inactivation of terminal complement components or anticoagulant therapy with heparin.

This work unit was recently reactivated to investigate further the vascular lesions of life-threatening rickettsial infections. Studies are now in preliminary stages.

Publication:

Moe, J. B., G. L. Ruch, R. H. Kenyon, J. D. Burek, and J. L. Stookey. 1976. Pathology of experimental Rocky Mountain spotted fever in rhesus monkeys. *Vet. Pathol.* 13:69-77.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^b	2. DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL	
3. DATE PREV SURVEY 75 07 01	4. KIND OF SUMMARY K. COMPLETION	5. SUMMARY SECY ^b U	6. WORK SECURITY ^b U	7. REGRADING ^b NA	8. DISEIN INSTRN NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT
10. NO./CODES: ^b	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
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c. <i>14/14/14/14/14/14</i>	CARDS 114(e)(f)						
11. TITLE (Provide with Security Classification Code) ^b (U) Pathogenesis of Rocky Mountain spotted fever in the rhesus monkey							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^b 003500 Clinical medicine; 004900 Defense; 002600 Biology (Pathology)							
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g. KIND OF AWARD:				21. PERFORMING ORGANIZATION			
19. RESPONSIBLE DOD ORGANIZATION		NAME: ^b USA Medical Research Institute of Infectious Diseases ADDRESS: ^b Fort Detrick, MD 21701		NAME: ^b Rickettsiology Division USAMRIID ADDRESS: ^b Fort Detrick, MD 21701			
RESPONSIBLE INDIVIDUAL		NAME: ^b Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Provide DSN // U.S. Academic Institutions) NAME: ^b Sammons, L. S. TELEPHONE: 301 663-7465 SOCIAL SECURITY ACCOUNT NUMBER:			
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22. WORDS (Provide each with Security Classification Code) (U) Pathogenesis; (U) Rocky Mountain spotted fever (RMSF); (U) Rickettsia; (U) Laboratory animals; (U) Military medicine; BW Defense							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Provide text of each with Security Classification Code.) 23 (U) To define the pathogenesis of RMSF in the rhesus monkey model. To use this information to improve planning for medical defense against potentially important rickettsial BW agents. 24 (U) Examine the pathophysiology and pathogenesis of RMSF in order to study prophylaxis and therapy. 25 (U) 75 07 - 76 06 - Blood acid-base changes were measured in RMSF-infected rhesus monkeys and they were found to demonstrate respiratory alkalosis. Arterial and venous blood pH were significantly increased while venous pCO ₂ was significantly decreased (p less than 0.01) during peak infection. Serum components were also measured in these monkeys and copper, cortisol, free fatty acids, triglycerides, glycoprotein, and haptoglobin were increased while albumin decreased. There was no change in cholesterol. Cynomolgus monkeys were assessed for their susceptibility to RMSF. They seemed to have a similar clinical response to rhesus monkeys except that the incidence of rash in the cynomolgus monkey was very low. An attempt was made to determine if there were other species of laboratory animals more susceptible to the spotted fever group of rickettsiae than the Hartley strain guinea pig and monkey. Six strains of mice, strain 13 guinea pigs, gerbils, ferrets, cotton rats, wild rabbits, sheep, miniature swine and voles were inoculated with various species of spotted fever rickettsiae. There was 100% mortality in Swiss Webster and BALB/C mice after inoculation with Rickettsia akari, and 50-75% mortality of voles after infection with the 2 Rickettsia species siberica and conorii. The investigator has left the Army. The study is complete. Publications: Am. J. Vet. Res. 37:725-731, 1976. J. Clin. Microbiol. 4: 253-257, 1976.							
26. Available to contractors upon originator's approval.							

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 01 301: Pathogenesis of Rocky Mountain Spotted Fever in the Rhesus Monkey

Background:

Rocky Mountain spotted fever (RMSF) is the most prevalent rickettsial disease in the United States. Pathologic lesions seen in RMSF in humans have been reported to be similar to those seen in the monkey infected with Rickettsia rickettsii.¹ Other investigators have reported that monkeys exposed to aerosolized R. rickettsii exhibit clinical signs which compare closely to those seen in man.² Because of the similarity of signs and lesions between man and monkey, and since eradication of rickettsiae in endemic areas is thought to be improbable, the nonhuman primate serves as a useful model to study pathophysiology and vaccine protection.

Studies at USAMRIID have defined the rhesus monkey infected with R. rickettsii in terms of clinical signs, hematologic data, coagulation and complement values.³ Because of the generalized nature of the lesions of RMSF, pathophysiologic studies have been continued to elucidate the disease process.

A shortage of rhesus monkeys has necessitated consideration of other species of monkeys and other types of animals susceptible to R. rickettsii. At the same time, emphasis on the other members of the spotted fever group of Rickettsia such as conorii, sibericus, and akari requires development of animal models for these organisms.

Progress:

The group of 16 rhesus monkeys used to determine the LD₅₀⁴ for R. rickettsii challenge studies were bled regularly to measure blood acid-base changes and additional serum components that had not been measured previously. All but one of the 16 monkeys became ill; only ill monkeys are included in the data in Table I. Arterial and venous blood pH were significantly increased ($P < 0.01$) while venous blood pCO₂ was significantly decreased ($P < 0.01$) during peak infection. On one day, arterial pCO₂ was increased significantly ($P < 0.01$). No significant changes were observed in arterial and venous blood pO₂ and HCO₃⁻. The ill monkeys demonstrated respiratory alkalosis.

TABLE I. ACID-BASE ALTERATIONS AND RECTAL TEMPERATURES IN RHESUS MONKEYS THAT DEVELOPED FEVER DURING R. RICKETTSII INFECTION

DAY	NO. OF MONKEYS STUDIED	RECTAL TEMPERATURE °C	pH		pO ₂ mm Hg		pCO ₂ mm Hg		HCO ₃ ⁻ mEq/l	
			Art.	Ven.	Art.	Ven.	Art.	Ven.	Art.	Ven.
-4	15	39.3	7.33	7.10	100.0	28.0	24.4	59.2	11.2	18.4
-1	15	39.2	7.34	7.04	102.5	31.4	25.1	64.8	11.6	17.2
1	15	39.2	7.36	7.15	102.4	29.0	26.5	53.4	13.1	18.4
3	15	39.3	7.38	7.20	96.0	33.7	33.9 ^a	55.0	17.8	20.6
5	15	40.8 ^a	7.38	7.19	105.3	27.6	22.8	45.7 ^a	12.6	17.4
6	15	40.6 ^a	7.46 ^a	7.30 ^a	92.0	24.6	24.0	34.0 ^a	14.7	16.7
7	15	40.6 ^a	7.50 ^a	7.38 ^a	103.2	24.5	20.3	31.8 ^a	14.0	19.0
8	12	40.3 ^a	7.52 ^a	7.42 ^a	103.0	23.4	21.7	29.0 ^a	14.9	18.6
9a.m.	10	40.4 ^a	7.47 ^a	7.27 ^a	95.3	25.8	22.0	42.0 ^a	14.4	19.4
9p.m.	9			7.30 ^a		17.4		38.0 ^a		17.5
10	6	39.4	7.32	7.30 ^a	109.6	26.9	30.8	33.9 ^a	13.1	18.1
11	4	39.3		7.24		32.9		42.3		17.8
12	4	39.2		7.23		28.6		38.9		16.2
13	3		7.45	7.20	81.6	25.7	30.0	56.0	18.8	21.6
15	3		7.33	7.10	99.2	35.8		52.2	15.4	15.4

^aP<0.01 compared to preinfection days -4 and/or -1, by Analysis of Variance.

Of those serum components measured, cholesterol did not change, while Cu, cortisol, free fatty acids, triglyceride, glycoprotein and haptoglobin increased above the normal limits. Albumin was decreased below the normal limits of the monkeys (Table II).

TABLE II. EFFECT ON SERUM COMPONENTS OF R. RICKETTSII INFECTION
IN 15 OF 16 RHESUS MONKEYS

COMPONENT	% SHOWING CHANGE	PREINFECTION MEAN \pm SD	NORMAL LIMITS OF THESE MONKEYS
<u>Increase</u>			
Cu	93	120.8 \pm 19.1 $\mu\text{g}/\text{dl}$	82-159
Cortisol	73	9.3 \pm 5.3 $\mu\text{g}/\text{dl}$	20
<u>Free Fatty Acids</u>			
	80	209.7 \pm 92.7 mEq/l	24-398
Triglycerides	93	34.8 \pm 14.0 mg/dl	7-63
Glycoprotein	100	<10% of SD	
Haptoglobin	80	23.0 \pm 9.4% of SD	<10-42
<u>Decrease</u>			
Albumin	87	38.4 \pm 4.3% of SD	30-47
<u>No change</u>			
Cholesterol		164 \pm 28.1 mg/dl	108-220

A study was initiated to examine RMSF in cynomolgus monkeys; 13 monkeys of random sex weighing 1.6 - 2.6 kg and with negative immunofluorescent titers to R. rickettsii (Sheila Smith strain) on WI-38 cells, were divided into 4 groups; 3 cynomolgus served as controls. All but the controls were inoculated SC with 1 ml of yolk-sac-grown R. rickettsii containing 10^1 , 10^2 , 10^3 or 10^4 PFU/ml. Clinical responses can be seen in Table III.

TABLE III. CLINICAL RESPONSE OF CYNOMOLGUS MONKEYS TO RICKETTSIA RICKETTSII INFECTION

DOSE	ILL/TOTAL	MEAN DAYS INCUBATION (RANGE)	MEAN DAYS DURATION OF FEVER (RANGE)	MTD (DAYS)	NO. DEAD
10^4	3/3	4	5	8	2
10^3	3/3	3 (2-4)	7.3 (5-9)	10	2
10^2	4/4 ^a	6 (4-8)	5.2 (5-6)	8	1
10^1	3/3	6 (5-7)	5.7 (4-7)	11	3
Controls	0/3	0	0	0	0

^aOne monkey had rash.

The criteria of fever (≥ 104 F) used in the rhesus monkeys was not adequate for the cynomolgus monkeys. The cynomolgus monkeys were ill (determined by demonstrable anorexia and lethargy) before rectal temperatures reached 104 F. Mean base-line rectal temperature for these 16 monkeys was 101.79 ± 0.75 . In an earlier study using cynomolgus monkeys (MAJ D. Harrington, USAMRIID), the beginning of fever was arbitrarily defined as 103.1 F. When 2 SD are added, our defined mean baseline temperature equals 103.29 F which agrees well with that defined earlier. Thus, for this study > 103.1 F was chosen to define fever. Of note was the absence of rash in ill cynomolgus monkeys; 50-75% of rhesus monkeys with RMSF generally show rash.

Data for mean days incubation, MTD and number of dead cynomolgus monkeys were similar to rhesus monkeys with RMSF. The mean days duration of fever appeared to be slightly longer in the cynomolgus at the 10^3 dose than in the rhesus. The other doses elicited similar responses in both species.

Rickettsiae were isolated from all but one febrile animal. There was a slight decrease in hematocrit of the control animals due to the regular bleeding procedures. The hematocrit for the ill animals decreased days 6-12 more than the control animals, which corresponds to that seen in rhesus monkeys. WBC counts in the febrile surviving monkeys were higher than the afebrile monkeys during days 12-20 which compares to days 8-15 in the rhesus. Most of the increase in WBC was accounted for by an absolute lymphophilia and slight

neutrophilia. On day 8 prior to the lymphophilia the febrile surviving monkeys had lymphopenia. This response is different from the febrile surviving rhesus which show an absolute neutrophilia and no difference in the lymphocytes from the afebrile rhesus monkeys.

Febrile cynomolgus monkeys that died showed a slight increase in WBC on day 2 which began to decrease during infection continuously until death. The slight increase in WBC was due to an absolute neutrophilia which continued until day 6 and then decreased. At the same time lymphocytes remained at base line through day 2 and then decreased so that lymphopenia existed from day 4-10. Febrile rhesus that died had little or no change in WBC and neutrophils but showed marked lymphopenia days 6-10. Platelets in febrile cynomolgus monkeys showed no difference from the afebrile while febrile rhesus which died had a definite thrombocytopenia.

Postmortem examination showed little difference between species except the spleens of cynomolgus monkeys were more enlarged than those of rhesus, and evidence of rash was decreased in the former.

Hartley and Strain 13 guinea pigs were inoculated SC with spotted fever group organisms, R. rickettsii (SS and Iowa), R. australis, R. conorii, R. siberica, and R. akari (Table IV). After infection with R. rickettsii (SS strain), Strain 13 guinea pigs responded with a mean 2.1 degree-days, whereas the Hartley strain guinea pigs showed 5.6. No Strain 13 guinea pigs died while 4 of 6 Hartley guinea pigs did. Strain 13 guinea pigs were not febrile in response to R. rickettsii (Iowa), R. akari, R. conorii or R. australis; however, these animals attained 0.8 degree-days with R. siberica. In contrast, Hartley strain guinea pigs were found to have 4.1, 1.6, 2.5, and 2.8 mean degree-days with R. akari, R. conorii, R. siberica and R. australis, respectively.

TABLE IV. GUINEA PIGS RESPONSE TO SPOTTED FEVER GROUP RICKETTSIAE

<u>RICKETTSIA</u> SPECIES (Strain)	DOSE PFU	MEAN DEGREE DAYS ^a	
		Strain 13 (n=2)	Hartley (n=6)
<u>R. rickettsii</u> (SS)	1.0×10^6	2.1	5.6 ^b
<u>R. rickettsii</u> (Iowa)	1.0×10^6	0	---
<u>R. akari</u>	2.5×10^6	0	4.1
<u>R. conorii</u>	2.5×10^5	0	1.6
<u>R. siberica</u>	1.5×10^6	0.8	2.5 ^c
<u>R. australis</u>	2.0×10^6	0	2.8

^a Sum of degrees >103.8 F in any one group per total number guinea pigs in that group.

^b 4 died.

^c n=5.

Six strains of mice and gerbils were infected IP and SC, respectively, with 5 members of the spotted fever group of rickettsiae; R. rickettsii (Sheila Smith strain), R. australis, R. conorii, R. siberica, and R. akari. The mice were inoculated with 0.5 ml yolk-sac-grown rickettsiae and the gerbils with 1.0 ml.

The results obtained from mouse strains inoculated with R. rickettsii, R. akari, R. conorii, R. siberica, and R. australis are presented in Table V. One death occurred in both the BALB/C and C57BL/65 mice due to infection with R. rickettsii. R. akari killed 100% of the BALB/C and Swiss Webster mice, only one death occurred in the DBA1 and DBA2 groups and no C57BL/65 mice died. Three Swiss Webster mice died from R. siberica and one AKR mouse and 3 BALB/C mice died from R. australis infection. No mice died from R. conorii infection. Neither of 2 gerbils became clinically ill and no deaths occurred.

TABLE V. MOUSE AND GERBIL DEATH RESPONSE TO SPOTTED FEVER GROUP RICKETTSIAE

MOUSE STRAIN	NO. DEAD/5 (DOSE, PFU)				
	<u>R. rickettsii</u> (SS) (1×10^6)	<u>R. akari</u> (2.5×10^6)	<u>R. conorii</u> (2.5×10^5)	<u>R. siberica</u> (1.5×10^6)	<u>R. australis</u> (2.0×10^6)
C3H	0	0	0	0	0
DBA1	0	1	0	0	0
DBA2	0 ^a	1	0	0	0
AKR	0	0	0	0	1
BALB/C	1	5	0	0	3
C57BL/65	1	0	0	0	0
Swiss Webster	0	5	0	3	0

^an=4.

Six male castrated ferrets (*Mustelo furo*), 6-12 months old, were inoculated SC with 1 ml of 10^3 , 10^5 , or 10^7 PFU/ml yolk-sac-grown R. rickettsii. Intermittent febrile periods were observed in all ferrets but one (10^7 PFU/ml inoculum) which had fever for only one day.

Ferrets inoculated with R. rickettsii showed 1.2 to 2.8 average degree days (Table VI) which was inversely proportional to the inoculum used. The only sign of clinical illness was slight lethargy during peak fever. At no time were ferrets totally anorectic. There was no evidence of rash. Isolation of rickettsiae by plaquing was unsuccessful. Total WBC, neutrophils and lymphocyte counts were normal to slightly increased.

TABLE VI. FERRET RESPONSE TO R. RICKETTSII

DOSE PFU	MEAN DEGREE F DAYS
10^3	2.8
10^5	2.7
10^7	1.2

Six weanling cotton rats inoculated IP with 0.5 ml of either 10^7 or 10^3 PFU/ml yolk-sac-grown R. rickettsii exhibited no signs of clinical illness. One rat infected with 10^7 PFU died 3 days postinoculation; however, this appeared to be an incidental death. Surviving cotton rats remained healthy in appearance and developed microagglutination (MA) titers ranging from 1:128 to 1:512.

Four wild rabbits (Sylvilagus sp.), approximately 4 months old, were inoculated SC with 1 ml of 10^3 or 10^7 PFU/ml yolk-sac-grown R. rickettsii. None of the rabbits showed any signs of illness. At 30 days postinoculation the rabbits were killed and MA titers measured. MA titers ranged from 1:128 to 1:512. Three wild rabbits approximately 4 months old were inoculated SC with 1 ml of 10^7 PFU/ml R. conorii; there were no signs of clinical illness, but one rabbit died on day 6. MA titers of rabbits inoculated with R. conorii ranged from 1:16 to 1:64.

One young sheep inoculated with 10^7 R. rickettsii displayed no signs of clinical illness nor rickettsemia. A second sheep inoculated with 9.2×10^7 PFU chick embryo cell-culture grown (CEC) R. rickettsii developed 5 days of fever but no other signs of clinical illness nor rickettsemia.

Miniature swine inoculated with yolk-sac or CEC grown R. rickettsii exhibited no signs of clinical illness. Blood cell counts remained within the normal range and rickettsemia was not detected. One pig exhibited a reddened, erythematous area surrounding the inoculation site on day 5.

In October, 78 voles (Microtus pennsylvanicus) of random sex and approximately 3 months of age were obtained from NIH (courtesy of M. Bozeman) to initiate a vole colony, since these animals have been reported to be susceptible to the spotted fever group of rickettsiae. During November approximately 75% of the voles died from an acute or chronic respiratory infection. Klebsiella pneumoniae

was isolated and may have been the causative organism. Retroorbital abscesses were also noted, possibly due to previous bleedings. Antibiotic therapy added to drinking water gave marginal success. Although several litters were born, deaths continued to occur among the adults and young.

As these voles were unsatisfactory for breeding purposes, they were used for spotted fever susceptibility testing. Six groups were inoculated IP with ~0.5 ml of 10^6 or 10^3 PFU/ml of yolk-sac-grown *R. rickettsii*, *R. siberica* or *R. conorii*. Results are shown in Table VII. Voles that died were submitted for histopathologic examination. Data are preliminary, since the voles were in doubtful condition. Subsequent studies will utilize SC injection. Nevertheless, the vole represents a potential model for susceptibility testing for the spotted fever microbes. Additional voles have been obtained through the courtesy of Dr. W. Burgdorfer from the Rocky Mountain Laboratories, Hamilton, Montana.

TABLE VII. RESPONSE OF VOLES TO 3 SPOTTED FEVER GROUP RICKETTSIAE

SPECIES (PFU/ml)	NO. DEAD/TOTAL	DAY OF DEATH
<i>R. rickettsii</i> (SS)		
1.0×10^3	0/4	
1.1×10^6	3/4	3,4,5
<i>R. siberica</i>		
4.0×10^3	3/4	3,3,5
4.0×10^6	2/4	6,7
<i>R. conorii</i>		
4.0×10^3	1/4	5
4.0×10^6	2/4	5,5

COLLABORATIVE STUDIES

A. Four rhesus monkeys which had been inoculated SC 4 months prior with yolk-sac-grown *R. rickettsii* (SS) were irradiated with 400 R of x-radiation to determine whether there was persistence of rickettsiae in monkeys after recovery from clinical infection (Work Unit 834 01 010). Rectal temperatures were recorded daily and blood was drawn for CBC every other day for 2-1/2 wk. None of the monkeys showed signs of RMSF; rectal temperatures remained normal. The WBC counts reflected the effect of irradiation by decreasing and slowly returning to normal. Results of this study indicate a lack of persistence of organisms and no recrudescence of disease.

B. A cooperative study with Dr. Liu (Work Unit 834 01 110) was initiated to study the feasibility of using the chaired rhesus monkey infected with R. rickettsii to study body fluid and tissue ion levels during RMSF. Four infected monkeys and 2 sham-inoculated controls were studied. To monitor infection in monkeys the following parameters were measured before and during infection: plasma urea nitrogen, hemoglobin, hematocrit, RBC and WBC. These parameters in the infected monkeys followed the RMSF rhesus model previously established. It appeared that chaired, infected monkeys ceased eating earlier in the course of illness and that the duration of illness was shortened by a few days. The incubation period (day of inoculation until first day of fever) was also accelerated.

Publications:

1. Sammons, L. S., R. H. Kenyon, G. T. Burger, C. E. Pedersen, Jr. and R. O. Spertzel. 1976. Changes in blood serum constituents and hematologic parameters in Macaca mulatta infected with Rocky Mountain spotted fever. Am. J. Vet. Res. 37:725-731.
2. Sammons, L. S., R. H. Kenyon, and C. E. Pedersen, Jr. 1976. Effect of vaccination schedule on immune response of Macaca mulatta to cell-culture grown Rocky Mountain spotted fever vaccine. J. Clin. Microbiol. 4: in press.

LITERATURE CITED

1. Lillie, R. D. 1941. Pathology of Rocky Mountain Spotted Fever. National Institutes of Health Bulletin No. 177. U.S. Government Printing Office, Washington, D. C., 59 pp.
2. Saslaw, S., and H. N. Carlisle. 1966. Aerosol infection of monkeys with Rickettsia rickettsii. Bacteriol. Rev. 30:636-645.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^b DA OF6419	2. DATE OF SUMMARY ^b 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
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29. TECHNICAL OBJECTIVE, 30. APPROACH, 31. PROGRESS (Printed individual paragraphs identified by number. Printed out of each with Security Classification Code.) 23 (U). Determine the role of macrophages as a host defense mechanism against infectious disease and to investigate means of enhancing host defenses by stimulation of the macrophage system or modification of cellular and humoral immune responses. This research is essential for developing approaches to successful immunoprophylaxis against this recognized BW threat and the treatment of casualties in BW operations. 24 (U) Starting with normal and immune peritoneal macrophage cultures and tissue-culture grown C. burnetii, examine mechanisms of phagocytosis; then conduct similar studies with alveolar macrophages.							
25 (U) 75 05 - 76 06 - The interaction between C. burnetii and peritoneal macrophages from immune and nonimmune guinea pigs was studied by light and transmission electron microscopy. Phase I rickettsiae were more resistant to phagocytosis than phase II organisms. Phase I and II organisms previously treated with normal serum multiplied within phagosomes of macrophages from normal and phase II immune animals. Phase I organisms were degraded by macrophages from phase I immune animals. Suspending rickettsiae in immune serum rendered the organisms more susceptible to phagocytosis, and also potentiated their destruction by macrophages. The fate of intracellular rickettsiae was not affected by homologous immune serum or chloramphenicol in the medium. Macrophage inhibition factor was detected in guinea pigs immunized with phase I or II killed C. burnetii antigens.							
Publication: Infect. Immunity 14: 1087-1096, 1976.							
D. available to contractors upon contractee's request.							
DD FORM 1 MAR 68 1498							PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.
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BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infections of Military Importance

Work Unit No. 834 01 303: Role of Macrophages in Respiratory Diseases

Background:

Many epidemics of Q fever involving thousands of soldiers in Europe during World War II has given evidence that this disease is a significant military problem.

The pathogenesis of Q fever and the mechanisms of immunity are not well known. The role of macrophages has been implicated, but the mechanisms by which they perform their function has not been fully explained. Downs¹ found that phase II rickettsiae were more resistant to phagocytosis by normal guinea pig peritoneal macrophages than were phase I organisms; whereas Kazár et al.² concluded the reverse to be true. The fate of ingested rickettsiae was not resolved by these investigators.

The ability of normal and immunized guinea pig peritoneal macrophages to phagocytize and kill ingested rickettsiae was studied by light microscopy. The results were substantiated by studying the interaction by transmission electron microscopy (TEM) so that direct visual observation could be obtained.

Progress:

Initial efforts were directed toward developing and standardizing techniques for processing large numbers of guinea pig peritoneal macrophages, growth of phase I and II Coxiella burnetii, production of antisera, and use of various stains. Also, several factors such as serum concentration in the medium, interaction time, effect of pretreatment of rickettsiae, and multiplicities of infection were investigated so that reliable and reproducible results could be obtained. Rickettsiae were grown in chick fibroblast cells, and the ID₅₀ was determined as previously described.³ Homologous immune sera were obtained from guinea pigs.³ Guinea pig peritoneal macrophages were harvested and processed according to the method of David et al.⁴ A multiplicity of 100 rickettsiae (previously treated with 10% normal or homologous heat-inactivated immune serum) per macrophage was used throughout the experiment. The macrophage-rickettsiae suspension was incubated for 60 min at 37 C; the phagocytic uptake and subsequent fate of ingested rickettsiae were determined as previously described.^{3,5}

Phagocytosis of C. burnetii by macrophages. The phagocytic uptake of phase I and II rickettsiae by macrophages from immune and nonimmune guinea

pigs in selected sera is summarized in Table I. Phase I rickettsiae treated with normal serum were significantly more resistant to phagocytosis by the different types of macrophages than phase II organisms ($P < 0.001$). The average number of phase I rickettsiae/infected cell was less than that of phase II organisms ($P < 0.001$). When phase I and II organisms were treated with homologous antiserum prior to interaction significantly more macrophages from immune and nonimmune animals contained rickettsiae ($P < 0.05$).

TABLE I. PHAGOCYTOSIS OF C. BURNETII BY DIFFERENT TYPES OF MACROPHAGES IN SELECTED SERA

TYPE MACROPHAGE	TYPE SERA	% PHAGOCYTOSIS		AVERAGE NUMBER OF RICKETTSIAE/INFECTED CELL	
		Phase I	Phase II	Phase I	Phase II
Normal	Normal ^a	7	80	4	20 ^b
	Immune	69 ^b	98 ^c	7	41
Phase I immune	Normal ^a	12 ^b	82 ^c	3 ^b	27 ^d
	Immune	93	92	34	40
Phase II immune	Normal ^a	10 ^b	84 ^b	5 ^b	28
	Immune	89	97	20	43 ^c

^a $P < 0.001$, Phase I vs. Phase II

^b $P < 0.001$, Immune vs. Normal

^c $P < 0.05$, Immune vs. Normal

^d $P < 0.02$, Immune vs. Normal

Fate of ingested rickettsiae. The fate of phase I ingested rickettsiae in macrophages is summarized in Table II. There was approximately a 12-fold increase in numbers of normal serum-treated phase I organisms within infected macrophages 5 days after ingestion. When the same organisms were ingested by macrophages from phase II immune animals, the ingested rickettsiae also multiplied. However, phase I organisms were destroyed after being ingested by macrophages from phase I immune animals. Phase II rickettsiae previously treated with normal serum multiplied within normal macrophages as well as macrophages from phase I or II immune animals. In contrast, antibody-treated phase I and II rickettsiae were degraded within macrophages from normal and immune animals (Table II).

TABLE II. FATE OF PHASE I AND II C. BURNETII AFTER INGESTION BY DIFFERENT TYPES OF MACROPHAGES IN SELECTED SERA

TYPE OF MACROPHAGE	TYPE SERUM	MEAN NO./INFECTED CELL BY DAYS				
		0	1	2	3	5
Phase I						
Normal	Normal	4	3	7	8	50
	Immune	7	8	5	6	0
Phase I immune	Normal	3	3	3	0	0
	Immune	34	25	14	4	0
Phase II immune	Normal	5	6	9	10	19
	Immune	20	17	8	3	0
Phase II						
Normal	Normal	20	40	45	45	50
	Immune	41	17	19	15	0
Phase I immune	Normal	27	39	42	43	44
	Immune	40	29	19	14	5
Phase II immune	Normal	28	40	44	46	49
	Immune	43	33	24	23	4

Specificity of macrophage-rickettsiae interaction. Additional experiments were performed to determine the specificity of macrophages from phase I immune animals to phase I rickettsiae. Macrophages from these animals as well as from normal animals were interacted with an unrelated rickettsia, Rickettsia rickettsii. After the 60-min interaction period, rickettsiae multiplied in both types of macrophages with subsequent destruction of the macrophages by 3-5 days.

Action of chloramphenicol and homologous antiserum on the fate of ingested rickettsiae. Experiments were performed to determine the effect of antibiotic or homologous immune serum on the fate of phagocytized rickettsiae. After normal serum-treated rickettsiae were phagocytized by macrophages, cells were washed 3 times with HBSS; 10 µg/ml chloramphenicol or 10% homologous antiserum in 1 ml Earle's 199 medium was added. Results indicate that neither chloramphenicol nor homologous immune serum had any effect on the intracellular rickettsiae or their subsequent replication.

Interaction study with TEM. Because of the small size of rickettsiae,

and the difficulty in quantitating death or growth, interaction studies between *C. burnetii* and normal guinea pig macrophages from normal and immune animals were studied. The results of the phagocytic uptake of rickettsiae and subsequent fate of ingested rickettsiae were based upon 4 replicates at each sample time. Approximately 100-200 cells were examined for each experimental treatment.

Examination of ultrathin sections confirmed our light microscopic observations and those of Kazár et al.² Phase I rickettsiae were more resistant to phagocytosis than phase II organisms. Approximately 2-5% of the macrophages contained 1-3 phase I rickettsiae, whereas 50-60% of the macrophages contained 5-10 phase II organisms when rickettsiae were pretreated with normal serum prior to interaction with macrophages. In contrast, pretreatment of rickettsiae with homologous immune serum enhanced the phagocytic uptake. Twenty to 30% of the macrophages contained 1-3 phase I organisms; 85-95% of the macrophages had 6-10 phase II organisms. The fate of antibody-treated and normal serum-treated rickettsiae were also similar to the light microscopic observations. Ingested rickettsiae were seen within well-defined macrophage phagosomes after the 60-min interaction period. Normal serum-treated rickettsiae multiplied freely in well-defined phagosomes for 2-3 days resulting in a 5- to 10-fold increase in the number of rickettsiae/infected macrophage.

An interesting morphological change in the rickettsia was seen within infected macrophages after ingestion of organisms treated with normal serum. Two forms were usually present; rod-shaped and round-to-oval organisms were observed. Oval forms lacked thick walls; their nucleoid filaments were more dispersed, and measured 0.4 by 0.8 μm ; round forms were similar to those seen when macrophages ingested antibody-treated rickettsiae after the 60-min interaction period. The appearance of the round type occurred later than with the antibody-treated organisms. A few were seen at 24 hr and many more at 48 and 72 hr. Some organisms were disrupted, with the cytoplasm contracted from the cell wall, indicating death of the organism. Pretreatment of phase I and II rickettsiae with immune serum not only enhanced their phagocytic uptake by macrophages, but also potentiated their destruction. No intact rickettsiae pretreated with immune sera were observed 1 day postinfection in the phagosomes of the macrophages.

Cell-mediated immunity (CMI) studies. Humoral immunity in man following infection or vaccination with *C. burnetii* is short-lived, yet recent work has shown blast cell transformation years later in seronegative individuals. Therefore, it appears that CMI is involved in Q fever. A positive macrophage inhibition factor (MIF) test has been used as an in vitro correlate of CMI. Studies were initiated to determine whether peritoneal exudate cells (PEC) obtained from guinea pigs immunized with killed, whole cell phase I or II *C. burnetii* would demonstrate MIF production. The direct MIF test using the agarose droplet technique was employed. Guinea pigs were given 4 weekly immunizations; the MIF test was performed 12-14 days after the last immunization. Results indicate that peritoneal macrophages from phase I immune animals are inhibited from

migration in the presence of soluble (trichloroacetic acid [TCA] extract) phase I or whole cell phase I and II antigens (Table III). This phenomenon was dose-related. Peritoneal macrophages from phase II immune animals were inhibited from migration only by phase II antigen, minimally by phase I particulate antigen at only the highest concentration, and not by soluble phase I antigen. This demonstrates a broad homologous and heterologous CMI response following phase I immunization, but only a narrow and weak homologous CMI response following phase II immunization.

TABLE III. MEAN MIGRATION INHIBITION OF PERITONEAL MACROPHAGES FROM GUINEA PIGS IMMUNIZED WITH PHASE I OR PHASE II C. BURNETII ANTIGEN

ANTIGEN	CONCENTRATION	% MIGRATION INHIBITION	
		Phase I Immunization	Phase II Immunization
Phase I TCA extract ^a	10 ⁻¹	24	2
	10 ⁻²	29	0
	10 ⁻³	22	0
Whole cell phase I <u>C. burnetii</u>	10 ⁸	42	20
	10 ⁷	34	0
	10 ⁶	4	0
Whole cell phase II <u>C. burnetii</u>	10 ⁸	TE ^b	34
	10 ⁷	21	15
	10 ⁶	5	0

^aCF titer = 1:512

^bTE = Toxic effect

Presentations:

1. Kishimoto, R. A. An electron microscopic study on the interaction between normal guinea pig peritoneal macrophages and Coxiella burnetii. Presented, Annual Joint Meeting - Maryland and Washington Branches of the American Society for Microbiology, Fort Detrick, Frederick, MD, 24 Apr 1976.

2. Kishimoto, R. A. Interaction between guinea pig peritoneal macrophages and Coxiella burnetii in vitro. Presented, 76th Annual Meeting, American Society for Microbiology, Atlantic City, N.J., 2-7 May 1976. (Abstracts of the Meeting - 1976, p. 11).

Publication:

Kishimoto, R. A., and J. S. Walker. 1976. Interaction between Coxiella burnetii and guinea pig peritoneal macrophages. Infect. Immun. 14: in press.

LITERATURE CITED

1. Downs, C. M. 1968. Phagocytosis of Coxiella burnetii, phase I and phase II, by peritoneal monocytes from normal and immune guinea pigs and mice. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. I: Orig. 206:329-343.
2. Kazar, J., E. Skultetyova, and R. Brezina. 1975. Phagocytosis of Coxiella burnetii by macrophages. Acta Virol. 19:426-431.
3. Kishimoto, R. A., B. J. Veltri, F. G. Shirey, P. G. Canonico, and J. S. Walker. 1976. An electron microscopic study on the interaction between normal guinea pig peritoneal macrophages and Coxiella burnetii. (Submitted for publication).
4. David, J. R., S. Al-Askari, H. S. Lawrence, and L. Thomas. 1964. Delayed hypersensitivity in vitro. I. The specificity of inhibition of cell migration by antigens. J. Immunol. 93:264-273.
5. Kishimoto, R. A., and J. S. Walker. 1976. Interaction between Coxiella burnetii and guinea pig peritoneal macrophages. Infect. Immun. 14: in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ¹ DA 0A6422	2. DATE OF SUMMARY ² 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SURVEY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY ³ U	6. WORK SECURITY ⁴ U	7. REGARDING ⁵ NA	8. DESCR INSTN ⁶ NL	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: a. PRIMARY 62 760A	PROGRAM ELEMENT	PROJECT NUMBER 3A762760A834		TASK AREA NUMBER 01	11. LEVEL OF SUM 401	
b. CONTRIBUTING						
c. CONFIDENTIAL CARDS 114(e)(f)						
12. TITLE (Printed with Security Classification Code) (U) Induced metabolic sequelae of infectious illnesses						
13. SCIENTIFIC AND TECHNOLOGICAL AREAS 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry						
14. START DATE 62 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house			
17. CONTRACT/GRANT		18. RESOURCES ESTIMATE	19. PROFESSIONAL MAN-YRS			20. FUNDS (in thousands)
a. DATES/EFFECTIVE: b. NUMBER: c. TYPE: d. KIND OF AWARD:		EXPIRATION: NA	FISCAL YEAR	76	1.0	135.7
		d. AMOUNT: e. CUM. AMT.	CURRENT	77	1.0	183.0
21. RESPONSIBLE DOO ORGANIZATION		22. PERFORMING ORGANIZATION				
NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		NAME: Physical Sciences Division USAMRIID ADDRESS: Fort Detrick, MD 21701				
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Printed with Security Classification Code) NAME: Powanda, M. C. TELEPHONE: 301 663-7181 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME:				
23. GENERAL USE Foreign intelligence considered		POC:DA				
24. KEYWORDS (Printed with Security Classification Code) (U) Laboratory animals; (U) Trace metals; (U) Amino acids; (U) Insulin; (U) Immunity; (U) Staphylococcus aureus; (U) Klebsiella pneumoniae; (U) Clofibrate; (U) BW defense						
25. TECHNICAL OBJECTIVE ⁷ AS APPROACH, IN PROGRESS (Printed individual paragraphs identified by number. Printed text of each with Security Classification Code.) 23 (U) Delineate the alterations in host metabolism which appear to occur as a direct consequence of host-parasite interaction and thus yield clues as to the pathogenesis of infection as well as act as prognostic indicators and indices of therapeutic effectiveness. Such studies are required to aid in, or devise new forms of, therapy for antibiotic-resistant bacterial as well as viral infections. Studies are designed to elucidate the metabolic needs of the severely infected soldier so as to minimize the period of convalescence.						
24 (U) Develop a number of model systems to study various aspects of the host-parasite interaction and host metabolism during inflammation and infection.						
25 (U) 75 07 - 76 06 - A model has been developed which may allow elucidation of some of the individual aspects of the host-parasite relationship as well as allow testing the efficacy of nonantibiotic/antiviral drugs and various forms of supportive therapy such as parenteral hyperalimentation, to minimize illness and aid patient recovery. Zinc deficiency in rats does not prevent the rats from synthesizing acute-phase globulins or immunoglobulins but appears to lead to decreased cell mediated immunity. Alterations in plasma lysozyme, alpha-2-macroglobulin and albumin are tested as valid indices of the effectiveness of antibiotic therapy in rats with respiratory K. pneumoniae infection. Clofibrate, an antihyperlipidemic drug, appears to have dose-dependent, differential effects on the synthesis and/or release of plasma proteins during inflammation. Clofibrate may thus be useful in dissecting out the function of various serum proteins during infection/inflammation.						
Publications: J. Cell Biol. 67:343A, 1975; Am. J. Physiol. 229:479-483, 1975; Biochem. Pharmacol. 25:785-788, 1976; Virology 70:241-243, 1976; Clin. Res. 24:351A, 1976; J. Nutr. 106:905-912, 1976; Proc. Soc. Exp. Biol. Med. 152:437-440, 1976; Acta Vitamin. Enzymol. 29:164-168, 1975; Am. J. Clin. Nutr. in press, 1976.						

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Aspects of Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 401: Induced Metabolic Sequelae During Infectious Illnesses

Background:

Induced metabolic sequelae (IMS) are alterations in host metabolism subsequent to the exposure of animals and man to a microorganism or a toxin and consequent upon phagocytosis of the microorganism, toxin or tissue damaged by them. The onset of certain metabolic changes appears to precede overt illness and thus can be considered amplifiers of the presence of the microorganism. Selected aspects of host metabolism may therefore have diagnostic import. Perhaps more important, other aspects of IMS appear to closely approximate the severity of the illness and thus may have prognostic value as well as allow one to monitor the effectiveness of various therapeutic regimens.¹⁻³ Finally IMS are indicative of the host-microorganism interaction and aid in elucidating pathogenesis of the illnesses.

Progress:

Part I.

In order to allow further elucidation of the host-microorganism interaction and to enable us to test regimens of therapy calculated to alter host response without concern as to their potential bacteriostatic or bactericidal activity *in vivo* we developed a model which so far appears to simulate bacterial infections. Rats were injected IP on day 0 with shellfish glycogen to stimulate the movement of leukocytes to the peritoneal cavity and then were given repeated IP injections of 10^{10} heat-killed staphylococci in saline to simulate the proliferation of bacteria associated with infection. Light microscopy indicated that 85-95% of the peritoneal leukocytes which accumulate on day 1 are neutrophils; by day 3 the population includes some 20-30% macrophages. Increasing numbers of staphylococci are observed to be associated with the PMN on days 2 and 3. Preliminary electron microscopic data indicate that the staphylococci are indeed ingested, not merely attached to the leukocytes. Repeated injections of heat-killed Staphylococcus aureus elicit significant decreases in plasma Zn, amino acids, free fatty acids, ketone bodies, and albumin concentration and increases in plasma Cu, seromucoid, haptoglobin, α_2 macrofetoprotein (α_2 -MFP), glucagon and insulin content and in the Phe/Tyr ratio as well as enhancing the flux of amino acids into liver. All of these alterations have been observed to occur in

men and animals with bacterial diseases. Heat-killed Staphylococcus epidermidis also elicit similar alterations in host metabolism eliminating the possibility that the alterations were occasioned by the enterotoxin associated with S. aureus. It is thus clear that many of the marked alterations in host metabolism which occur during infection are the result of phagocytic activity, i.e., sequelae to a primary host defensive mechanism, rather than the direct result of bacterial infection. Since heat-killed organisms are employed rather than agents which elicit sterile abscesses, this model may allow experiments of the effect of immunity on the host metabolic response to infection. Also since these injections do not necessarily lead to death, yet effect profound metabolic alterations, this system may allow us to simulate chronic infections and evaluate parenteral hyperalimentation under these circumstances.

Part II.

The interaction between Zn and host response to infection is proceeding in 2 directions: (1) the effect of its deficiency on the immune response to infection and (2) the effect of treatment with it on endotoxemia. The former approach is being carried out in collaboration with Dr. R. S. Pekarek, Human Nutrition Lab, USDA, Grand Forks, ND. Zn deficiency does not prevent rats from responding to infection (tularemia) with increased amounts of acute-phase globulins or to immunization (the live vaccine strain of Francisella tularensis) by developing normal or even somewhat increased levels of humoral immunity (total γ -globulin, IgG and specific agglutinating antibody). However, Zn deficiency decreased the in vitro lymphocyte response to phytohemagglutinin stimulation suggesting that the deficiency depressed the cell-mediated immune system.

LTC Sobociński (Work Unit 834 02 111) will report on the effects of Zn treatment on endotoxin lethality in rats.

We are also interested in the effect of Zn therapy on metabolism. Our initial observations indicate that zinc chloride in excess of 0.4 mg/100 gm body wt increases the amino acid uptake by liver. We are collecting data to see if amino acid concentrations and patterns in plasma, liver and muscle are affected.

Part III.

Our initial studies (collaboration with Dr. Berendt, Work Unit 834 02 109) of respiratory Klebsiella pneumoniae infection in the rat indicated that plasma seromucoid concentration correlated with the concentration of bacteria in the lungs; while plasma levels of α_1 -MFP and lysozyme did not increase markedly or plasma Zn values decrease until the concentration of Klebsiella in the lung approached 10^5 CFU/gm tissue, thus suggesting a threshold effect

in regard to the latter variables. In a latter study infected and control rats were bled sequentially from the orbital sinus; bacteremia, total WBC count and plasma seromucoid, Zn, lysozyme, and α_2 -MFP concentrations were determined. Infected rats that died had persistent leukopenia. Plasma Zn concentration decreased in all rats, probably as a result of the trauma of sampling. Infected rats that died, however, showed still greater decreases in the levels of plasma Zn. No changes in plasma lysozyme, α_2 -MFP and seromucoid were noted as a consequence of sampling. Infected rats which did not succumb to the illness displayed transient increases in these 3 variables. Infected rats that died from the disease displayed markedly greater increases in these 3 metabolic sequelae, which persisted until death. Dr. Berendt will comment on our findings in using plasma α_2 -MFP, lysozyme and albumin as indices of effectiveness of antibiotic therapy.

Part IV.

We have previously reported that clofibrate (p-chlorophenoxyisobutyrate, an antihyperlipidemic drug) protected some rats against a lethal infection produced by Streptococcus pneumoniae, inhibited the production of herpes simplex virus, type I, in human epithelial cell culture, and reduced the production of Pichinde virus (related to Machupo virus which causes BHF) in cell cultures. Clofibrate also reduces the plasma seromucoid concentration and increases that of albumin, an effect opposite to that of infection and inflammation. We therefore tested the effect of clofibrate on host responses to inflammation produced by SC injection of turpentine. (We resorted to studying a sterile abscess rather than an infection so as to simplify the interpretation of the data, since clofibrate has antibacterial activity *in vivo*.) Daily IM injections of clofibrate (140 mg/kg) begun 6 hr prior to the SC injection of turpentine did not prevent the decrease in albumin nor the rise in seromucoid. In regard to specific proteins, however, clofibrate significantly inhibited but did not prevent, the appearance of α_2 -MFP in the plasma (indicative of de novo protein synthesis) and the increase in plasma Cu (indicative of ceruloplasmin synthesis and/or release). This dose of clofibrate did not prevent the increase in haptoglobin concentration, the leukopenia, the decrease in plasma zinc or the increased uptake of amino acids by the liver, nor did it seem to diminish abscess formation. Larger doses of clofibrate completely block the appearance of α_2 -MFP and the increase in ceruloplasmin, but only mute the increase in haptoglobin. These data indicate that clofibrate may have dose-dependent, differential effects on the synthesis and/or release of plasma proteins. Thus clofibrate may be useful in dissecting out the function of various serum proteins during infection/inflammation.

Clofibrate also elicits decreases in plasma Zn and Cu concentrations. The latter's decrease appears to be associated with a lessened amount of

ceruloplasmin, the major Cu-transporting protein in blood. Other mechanisms may be operative as regards the decrease in plasma Zn. Clofibrate also markedly decreases plasma transferrin concentrations.

Presentations:

1. Powanda, M. C., and P. Z. Sobocinski. Systemic metabolic consequences of phagocytosis. Presented, Annual Meeting, American Society for Cell Biology, San Juan, PR, 11-14 November 1975. (J. Cell Biol. 67: 343a, 1975).
2. Pekarek, R. S., M. C. Powanda, and A. M. Hoagland. Effect of zinc deficiency on the immune response of the rat. Presented, Annual Meeting, FASEB, Anaheim, CA, 11-16 April 1976. (Fed. Proc. 35:360, 1976).

Publications:

1. Powanda, M. C., G. L. Cockerell, J. B. Moe, F. B. Abeles, R. S. Pekarek, and P. G. Canonico. 1975. Induced metabolic sequelae of tularemia in the rat: correlation with tissue damage. Am. J. Physiol. 229:479-483.
2. Powanda, M. C., E. L. Henriksen, E. Ayala, and P. G. Canonico. 1976. Clofibrate-induced alterations in serum protein patterns. Biochem. Pharmacol. 25:785-788.
3. Steinhart, W. L., C. S. Hogeman, and M. C. Powanda. 1976. Inhibition of the production of infectious herpes simplex virus by clofibrate. Virology 70:241-243.
4. Powanda, M. C., E. C. Hauer, R. E. Whitmire, J. P. Fowler, L. A. Harris, and R. H. Kenyon. 1976. Trace metal and lipid levels during Rocky Mountain spotted fever in the guinea pig. Clin. Res. 24:351A.
5. Powanda, M. C., and P. G. Canonico. 1976. Protective effect of clofibrate against S. pneumoniae infection in rats. Proc. Soc. Exp. Biol. Med. 152:437-440.
6. Pekarek, R. S., and M. C. Powanda. 1976. Protein synthesis in zinc deficient rats during tularemia. J. Nutr. 106:905-912.
7. Powanda, M. C., R. E. Dinterman, R. W. Wannemacher, Jr., and W. R. Beisel. 1975. Tryptophan metabolism in relation to amino acid alterations during typhoid fever. Acta Vitaminol. Enzymol. 29:164-168.
8. Powanda, M. C. 1976. Changes in body balance nitrogen and other key nutrients: description and underlying mechanisms. Workshop on Impact of Infection on Nutritional Status of the Host, Warrenton, VA, 11-13 May 1976. Am. J. Clin. Nutr., in press.

LITERATURE CITED

1. Powanda, M. C., G. L. Cockerell, J. B. Moe, F. B. Abeles, R. S. Pekarek, and P. G. Canonico. 1975. Induced metabolic sequelae of tularemia in the rat: correlation with tissue damage. Am. J. Physiol. 229:479-483.
2. Berendt, R. F., G. G. Long, P. G. Canonico, F. B. Abeles, and M. C. Powanda. 1976. Induced metabolic sequelae of respiratory Klebsiella pneumoniae infection in rats. (Manuscript in preparation).
3. Sabel, K.-G., and L. A. Hanson. 1974. The clinical usefulness of C-reactive (CRP) determinations in bacterial meningitis and septicemia in infancy. Acta Paediatr. Scand. 63:381-388.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSED ^a DA OC6428	2. DATE OF SUMMARY ^b 76 07 01	3. REPORT CONTROL SYMBOL DD-DR&E(AR)636
1. DATE PREV SURVEY 75 09 24	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCITY ^c U	6. WORK SECURITY ^c U	7. REGARING ^d NA	8. DOD/GEN INSTN ^d NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: ^e a. PRIMARY 62760A	PROGRAM ELEMENT PROJECT NUMBER 3A762760A834			TASK AREA NUMBER 01	11. LEVEL OF DOD & WORK UNIT 406	
b. CONTRIBUTING						
c. 114(e)(f)	CARDS 114(e)(f)					
12. TITLE (Proceed with Security Classification Code) (U) Analysis of subcellular structures in viral infections						
13. SCIENTIFIC AND TECHNOLOGICAL AREA ^f 003500 Clinical medicine; 004900 Defense; 002600 Biology						
14. START DATE 72 07	15. ESTIMATED COMPLETION DATE CONT	16. FUNDING AGENCY DA	17. PERFORMANCE METHOD C. In-house			
18. CONTRACT/GRAANT		19. RESOURCES ESTIMATE	20. PROFESSIONAL MAN YRS	21. FUNDS (in thousands)		
a. DATES/EFFECTIVE:		EXPIRATION:	FISCAL YEAR	1.0	83.7	
b. NUMBER: NA		C. AMOUNT:	CURRENT	1.0	230.0	
d. TYPE:		e. CUM. AMT.				
g. KIND OF AWARD:						
22. RESPONSIBLE DOD ORGANIZATION						
NAME: USA Medical Research Institute of Infectious Diseases			NAME: Pathology Division USAMRIID			
ADDRESS: Fort Detrick, MD 21701			ADDRESS: Fort Detrick, MD 21701			
23. RESPONSIBLE INDIVIDUAL						
NAME: Metzger, J. F. TELEPHONE: 301 663-2833			NAME: White, J. D. TELEPHONE: 301 663-7211 SOCIAL SECURITY ACCOUNT NUMBER:			
24. GENERAL USE						
Foreign intelligence considered						
25. OBJECTIVES (Proceed with Security Classification Code) (U) Electron microscopy; (U) BW defense; (U) Military medicine; (U) Ultrastructure; (U) Staphylococcal enterotoxin B; (U) Mycoplasma; (U) Arenaviruses; (U) Typhus						
26. TECHNICAL OBJECTIVE, 26. APPROACH, 26. PROGRESS (Proceed with Security Classification Code)						
23 (U) Study infections and toxic states at the ultrastructural level by scanning and transmission electron microscopy so as to elucidate mechanisms by which infectious microorganisms enter and leave cells and to identify target organelles damaged by microorganisms and toxins. These studies should provide basic information relative to specific therapy and protection against diseases caused by these agents and could lead to early detection of agents of potential BW importance.						
24 (U) Standard procedures for preparing biological material for scanning material were modified and adopted for examination of specimens in the scanning electron microscope. New methodology is developed where required to elucidate the pathogenic changes expressed by alterations to surface structures. Studies are correlated with conventional transmission microscopy.						
25 (U) 75 07 - 76 06 - Various cell cultures or tissues from animals exposed to pathogenic microorganisms were studied by scanning and transmission electron microscopy. Virions of Pichinde virus, seen on the surface of cells in culture for 60 min after application of the infecting dosage, adhere to the cell surface without distorting the plasma membrane. Rickettsia rickettsii enter cells in culture within 1 hr and are seen beneath the cell membrane. They are released through defects in the plasma membrane. Mycoplasma pneumoniae were seen on tracheal, bronchial and bronchiolar epithelium of Golden Syrian hamsters 7 and 14 days after intranasal instillation of the organisms. In addition, mycoplasma were seen on the surface of certain alveolar cells.						
Publications: Vet. Pathol. 13, in press, 1976. Lab. Invest. 35, in press, 1976.						

DD FORM 1498
MAR 1974PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

* U.S. GPO: 1974-840-843/8691

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 406: Analysis of Subcellular Structures in Viral Infections

Background:

Scanning electron microscopy (SEM) provides direct information about the topography of objects. Since the image has 3-dimensional quality which can be further enhanced by the examination of stereopairs, the spatial relationships between structures is more easily understood. Large numbers of cells in culture or *in situ* within organs can be examined quickly and with adequate resolution to recognize infectious microorganisms on the cell surface.

Progress:

Cell cultures and tissues or cells from animals exposed to pathogenic microorganisms were studied by conventional transmission electron microscopy (TEM) or SEM to establish basic morphologic data about interactions between cells and etiologic agents of disease, particularly events concerning entrance and exit of microorganisms. The present report deals with the effects of Pichinde virus, Rickettsia rickettsii, Mycoplasma pneumoniae and staphylococcal enterotoxin B (SEB).

We have previously reported and described the resolution of structure in 2 animal viruses, VEE and VSV through secondary electron image formation in the SEM. This report and others, more recent in the literature, demonstrate the utility of SEM in virological studies. Pichinde virus, a large pleomorphic arenavirus, was examined by SEM in an attempt to study cellular penetration and emergence by a virion. In order to determine if preparatory techniques for SEM were deleterious to the structure of the virion, negatively stained specimens and sections of embedded material prepared at various stages of SEM processing were examined by conventional TEM. There was no evidence of any gross alterations in either the internal ribosome-like granules or peplomers which are characteristic of this virus. Fetal rhesus monkey lung cells grown in culture on glass coverslips and aluminum foil, were infected with the virus at a high multiplicity (100:1). In samples obtained within 30 and 60 min after application of the infecting dosage, we were able to recognize virions on the surface of the cells in culture. By using

stereopairs, it was possible to see that the virus was adherent to the cell surface without distortion of the cell membrane. Although many virions were observed, there was no evidence of viral penetration at the times examined. At the end of 72 hr, the cells in culture appeared normal and the surface morphology was no different from that of uninfected cells incubated for a comparable length of time. However, virions were seen in the infected cell cultures. It is possible that this virus either replicates within many cells of the culture without cytopathic effects or within a few cells which are lysed and whose loss from the monolayer is not apparent. It is not clear why virions were not observed either penetrating or emerging through the surface of the cells. In addition to the possibility that the times for sampling were not appropriate, it is quite possible that this event was not recognized by morphologic appearance alone. Thus, it is apparent that the shape of the virion is not an adequate criterion for these studies and the use of an immunologic marker is required.

In addition to examining viral penetration of cells, a study of cellular infection by R. rickettsii was initiated. With one exception rickettsiae are obligate parasites and it is generally accepted that they are passive objects which gain entrance to cells by being ingested. The fibroblast is neither motile nor phagocytic in cell culture, therefore, it is an ideal cell to study the cellular rickettsial interactions during infection for comparison with the conditions that exist in cell penetration by viruses.

Cell culture of human fibroblasts (WI-38) were infected with R. rickettsii and prepared for TEM and SEM. Thus far, experiments have been designed to determine optimal times for obtaining samples to study. After placing rickettsiae on the cells, 1 hr is the earliest we have taken samples and it appears that by this time the organisms have already entered the host cell. Raised portions of the cell membrane outlining the intracellular rickettsiae are prominent; in many instances there is a long tubular extension of host cytoplasm which extends distally from one end of the rickettsia. Specimens obtained earlier than 1 hr have been prepared but have not been examined in the SEM at present. Examination by TEM reveals that prior to uptake of the rickettsiae there is a thickening of the plasma membrane at the point of contact between rickettsia and cell wall. By 24 hr rickettsiae are seen emerging from the cells. There appear to be at least 2 ways in which this occurs. In some instances rickettsiae have remained near the surface in a tubular fold of cell membrane and the organism is seen extending through a lateral split in the tube. Other rickettsiae are seen in large cavities in the cytoplasm which are open to the exterior through a break in the otherwise smooth surface of the cell. At 24 hr there does not appear to be much lytic damage to the cells, which are structurally intact. These findings will be substantiated with parallel TEM studies. The identity of the R. rickettsii has been confirmed in these observations by the use of specific antibodies bound to latex spheres.

M. pneumoniae, etiologic agent of a lower respiratory infection in man, is associated primarily with ciliated respiratory epithelium in its development. These studies were designed for the purpose of studying an experimental respiratory infection in hamsters by SEM. Prior ultrastructural studies by others have employed tracheal organ cultures. In preliminary studies prior to aerosol exposure of animals, Golden Syrian hamsters were administered $10^{7.6}$ M. pneumoniae, strain PI 1428, by nasal aspiration. Specimens of trachea and lung were prepared by either dehydration followed by critical point drying or by dehydration in vacuo from freeze-fractured samples. All material was sputter-coated with gold. In addition to the tissue, agar grown colonies of the mycoplasma were prepared for SEM to determine the appearance of the organism grown in an air atmosphere. These colonies were also treated with antibody-coated latex beads. The colonies were circular and consisted of tightly packed intertwined filaments with a diameter of 0.15 - 0.2 μm . The filaments terminate as cylinders with uniform diameter and round ends. The central portions of some large colonies were granular in appearance and devoid of filaments. Specific binding of antibody-coated beads was limited to the periphery of the colonies. This indicates an antigenic difference between the younger, log-phase mycoplasma at the edges and the static older area in the center. There was no binding of antibody-coated beads to agar.

Short stubby filaments which resemble the mycoplasma seen in agar culture were found consistently in trachea, bronchi and bronchioles of animals killed 14 days after exposure and with a lesser frequency in animals, at 7 days. Occasionally a solitary cell was seen in alveoli which appeared to be ciliated and covered with mycoplasma. These cells were seen only in 14-day animals and appeared to be *in situ* and were not epithelial cells which have been dislodged from higher in the respiratory tract. These mycoplasma appear to bind specific antibody-coated latex beads.

The mitogenicity of SEB for lymphocytes has been demonstrated by others in these laboratories. We have attempted to demonstrate binding of SEB to the surface of lymphocytes by using antibody-coated beads in the SEM. Specificity of the reaction was demonstrated using cells of Staphylococcus aureus and beads coated with antibody specific for SEB; however, it was not possible to show binding of SEB to thymic lymphocytes exposed to the toxin for 15 min.

These studies have been possible through the collaboration of the following investigators: CPT J. D. Gangemi, Pichinde virus; Mr. L. R. Bagley, Jr., R. rickettsii; Dr. J. V. Jemski and MAJ C. M. Hetsko, M. pneumoniae; and Dr. L. Spero, SEB binding.

Publications:

1. McLeod, C. G., J. L. Stookey, and J. D. White. 1976. Intestinal Tyzzer's disease, spirochetosis and cryptosporidiosis in a guinea pig. *Vet. Pathol.* 13: in press.
2. Moe, J. B., D. F. Mosher, R. H. Kenyon, J. D. White, J. L. Stookey, L. R. Bagley, and D. P. Fine. 1976. Functional and morphologic changes during experimental Rocky Mountain spotted fever in guinea pigs. *Lab. Invest.* 37: in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ² DA OD6416	2. DATE OF SUMMARY ³ 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
2. DATE PREV SURY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY ⁴ U	6. WORK SECURITY ⁵ U	7. REGARDING ⁶ NA	8. DOD/PN INSTRN ⁷ NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: ⁸ a. PRIMARY 62760A	PROGRAM ELEMENT PROJECT NUMBER 3A762760A834	TASK AREA NUMBER 01		11. LEVEL OF SUM A. WORK UNIT WORK UNIT NUMBER 407		
c. CONTRIBUTING / / / / /	CARDS 114(e)(f)					
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁹ 003500 Clinical medicine; 004900 Defense; 010100 Microbiology						
13. START DATE 72 08	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house			
17. CONTRACT/GRAANT		18. RESOURCES ESTIMATE	19. PROFESSIONAL MAN YRS	20. FUNDS (in thousands)		
a. DATES/EFFECTIVE: b. NUMBER: c. TYPE: d. KIND OF AWARD:		EXPIRATION: NA	FISCAL YEAR 76	1.0	110	
		AMOUNT: e. CUM. AMT.	CURRENT 77	1.0	229	
21. RESPONSIBLE DOD ORGANIZATION		22. PERFORMING ORGANIZATION				
NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		NAME: Virology Division USAMRIID ADDRESS: Fort Detrick, MD 21701				
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Punish DOD or U.S. Academic Institution) NAME: Jahrling, P. B. TELEPHONE: 301 663-7241 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME:				
23. GENERAL USE Foreign intelligence considered		POC:DA				
24. WORDS (Provide EACH with Security Classification Code) (U) Virulence; (U) Encephalitis, equine (VEE, WEE, EEE); (U) Interferon; (U) Reticuloendothelial system; (U) BW defense (U) Military medicine						
25. TECHNICAL OBJECTIVE, 26. APPROACH, 27. PROGRESS (Punish individual paragraphs identified by number. Provide last of each with Security Classification Code.)						
23 (U) Compare the pathogenesis for rodents of virulent and attenuated (vaccine) arbovirus strains, by identifying target tissues destroyed by virulent strains and by evaluating the protective responses induced by attenuated strains. Biochemical and biophysical properties of selected virus strains are correlated with biological parameters which determine degree of virulence.						
24 (U) Direct effects of virus replication in target tissues are assessed by a variety of means. Immune reactions to infection are compared, and the effects of immunosuppression examined. Key virus-to-cell interactions are studied in explant culture.						
25 (U) 75 07 - 76 06 - The mechanism which limits viremia in hamsters inoculated with benign strains of VEE, EEE, WEE, and SFV involves their efficient interaction with hepatic reticuloendothelial cells. This may depend on surface charge, since benign alphaviruses can be separated from virulent strains by hydroxylapatite chromatography. Efficient clearance of alphaviruses in other animal hosts, including guinea pigs, burros, and rhesus monkeys also correlates with low virulence; however in cotton rats, some VEE strains benign for this host are not efficiently cleared, indicating a different mechanism for limiting the infection.						
Publications: J. Infect. Dis. 132:667-676, 1975. J. Gen. Virol. 28:1-8, 1975. 32:121-128, 1976; Arthropod-borne Virus Information Exchange 30:137-140, 1976. Am. Soc. Microbiol. Abstracts of the Meeting -- 1976, p. 210. Arch. Virol. 51: in press, 1976.						

Available at Government Supply Centers and Contractors.

DD FORM 1 MAR 68 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

* U.S. GPO: 1974-840-843/8691

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 407: Comparative Pathogenesis of VEE Virus for Rodents
in Relation to Host Defense Mechanisms

Background:

We have reported that VEE strains which are benign for hamsters are rapidly removed from the circulation of hamsters while virulent strains are cleared slowly.¹ We demonstrated that one benign VEE virus adsorbed efficiently to Kupffer cells in the liver, while a virulent strain did not.² Further experiments, which constitute this report, were designed to determine if the correlation between efficient clearance and low virulence applied to other alphaviruses in the hamster, and to other animal hosts. In addition preliminary investigations to determine the biochemical basis for differences among virus strains in adsorption characteristics were initiated.

Progress:

Clearance and Distribution of Alphaviruses in Tissues of Hamsters. Alphavirus strains listed in Table I were grown in BHK-21 cells, intrinsically labeled with ^{32}P , concentrated and purified using reported procedures.² Clearance of virus from blood of hamsters inoculated via the intracardiac route was measured by assaying residual PFU and ^{32}P activity in plasma obtained 1, 5, and 30 min after inoculation; distribution of virus was measured by assaying ^{32}P activity in NaOH-digested tissues obtained 30 min after virus inoculation.²

TABLE I. VEE VIRUS STRAINS TESTED

HAMSTER-VIRULENCE ^a	
Lethal	Benign
VEE-Trinidad (LP) 69Z1 63U2	VEE-Trinidad (SP) TC-83 vaccine BeAr 35645 (Pixuna)
EEE - Arth 167 (LP)	EEE - Arth 167 (SP)
WEE - 72V1880 (LP) 72V4768	WEE - 72U1880 (SP) CM4-146
SFV - L ₁₀ H ₆ C ₁ /A	SFV-A774/C ₂ A

a. Lethal = killed all hamsters inoculated SC with 1000 PFU SC
Benign = killed < 15% of hamsters.

The following patterns were obtained. None of the 8 hamster-lethal strains were detectably cleared within 30 min of inoculation. In contrast, the benign strains were all rapidly cleared, and concentrated in the liver. Although minor differences in clearance rates were detected among the benign strains, in all cases > 99% of the input virus was cleared within 10 min; between 47% (for VEE-SP) and 73% (for WEE-SP) of the input virus could be accounted for in the liver. Differences in clearance rates did not depend on differences in aggregation since all virus activity (as measured by PFU and ^{32}P) passed a $0.10\text{-}\mu$ filter. The lack of aggregation was confirmed by electron microscopy for VEE-LP and SP, and for WEE-LP and SP. Clearance rates of virus as determined by PFU were identical with rates determined by ^{32}P assay, indicating that clearance reflected physical removal of virus from the blood, and not simply inactivation without subsequent removal. Rapid clearance did not correlate strictly with small plaque size, since benign VEE strain BeAr 35645 formed large plaques, while virulent VEE strain 69Z1 formed small plaques.

Elution of (^{32}P) Alphaviruses from Hydroxylapatite Columns Using Linear Phosphate Gradients. Hydroxylapatite chromatography separates proteins primarily on the basis of charge. Elution patterns can be dramatically affected by modifications in procedure. It is not known if the conditions employed (20 x 1.5 cm columns, pH 6.8; total buffer volume 300 ml) are optimal for separation of different alphaviruses. However, from Table II it is apparent that under the stated conditions, higher molarity phosphate buffer was required to elute the benign alphaviruses tested than their virulent counterparts. Furthermore, affinity for hydroxylapatite correlated closely with rapid clearance, and not with plaque size. These data suggest that the surface charge of benign alphaviruses is negative relative to the lethal strains.

TABLE II. PHOSPHATE MOLARITY OF ELUTION PEAKS CONTAINING MAXIMUM VIRUS ACTIVITY

VIRUS	PHOSPHATE MOLARITY	
	Lethal	Benign
WEE-LP	0.050	0.214
SP		
VEE-LP	0.153	0.194
SP		
69Z1	0.175	
TC-83		0.219
Pixuna		0.353

Other Applications of Hydroxylapatite Chromatography.

(a) The procedure was modified, as reported,³ to distinguish between epizootic and enzootic strains of VEE.

TABLE III. PHOSPHATE MOLARITY AND PLAQUE MORPHOLOGY OF ELUTION PEAKS

VEE SUBTYPE	VEE STRAIN	PHOSPHATE MOLARITY (plaque morphology) ^a	
		Major peak	Minor peak
I-A	Trinidad	0.43 (L)	0.10 (L)
I-B	69Z1	0.418 (S)	0.08 (L)
I-B	PTF-39	0.417 (S)	0.10 (L)
I-C	P676	0.430 (S)	0.05 (L)
I-D	3880	0.05 (L)	0.168 (MIX)
UNCLASSIFIED	Tumaco	0.05 (L)	0.400 (MIX)
I-E	68U201	0.05 (L)	"tails" (MIX)
I-D	Magangue	0.359 (MIX)	0.078 (L)

a. L= large plaques > 5 mm in diameter after 48 hr incubation.

S= small plaques < 2 mm in diameter after 48 hr incubation.

MIX= mixture of L and S plaques in approximately equal proportions.

The technique appears to be a potentially useful tool for characterizing new isolates of VEE, and for isolating subpopulations of virus from apparently homogeneous virus stocks.

(b) The heterogeneous nature of 2 avirulent WEE virus isolates, CM4-146 and CM4-977, was demonstrated.³

(c) Viruses isolated from the throat washings of 3 persons reacting to TC-83 vaccine could be distinguished from the vaccine on the basis of their elution characteristics. This suggests that the virus isolated from vaccine reactors is more virulent than the predominant vaccine virus population.

Clearance of VEE viruses from Blood of Other Animals. To determine if rapid clearance correlates with low virulence in rhesus monkeys, the clearance rates of (³²P) Trinidad VEE and TC-83 were compared, using described procedures.⁴ The data in Table IV demonstrate that TC-83 vaccine was cleared at a significantly faster rate than the Trinidad strain.

A similar study was done with burros using unlabeled virus. Epizootic VEE strain 69Z1, which is equine-virulent was compared with VEE strain 68U201, which is enzootic and equine-benign. Clearance of 68U201 was significantly more rapid than 69Z1 (Table V).

TABLE IV. CLEARANCE OF VEE STRAINS FROM THE BLOOD OF RHESUS MONKEYS^a
(n=3)

MIN	% OF INOCULUM REMAINING IN BLOOD			
	TC-83		Trinidad	
	PFU	³² P	PFU	³² P
1	6.9	6.6	98.0	98.1
5	2.1	1.9	79.0	80.1
10	1.2	1.5	69.0	67.2
30	1.2	0.9	37.0	40.5
60	0.1	0.2	31.0	37.3

a. Based on 3 monkeys for each virus.

TABLE V. CLEARANCE OF VEE STRAINS FROM THE BLOOD OF BURROS^a (n=2)

MIN	LOG ₁₀ PFU CLEARED	
	69Z1	68U201
1	0.10	0.80
2	0.82	1.10
20	1.00	1.35
30	1.20	1.59
60	1.25	2.36
120	1.50	2.86

a. Based on 2 burros for each virus.

These data are compatible with the low virulence and low level viremias associated with enzootic VEE strain infections of equines.

Clearance rates were also determined for 68U201 in Sigmodon hispidus (cotton rat), a reservoir host for VEE in nature. Strain 68U201, does not produce significant disease in cotton rats, although it does produce significant viremias (5-6 log₁₀ PFU/ml). In the clearance experiment, using 8 adult cotton rats, no virus was cleared during the 30-min test period. Other virus strains will be tested. However, it appears that the low virulence of VEE virus for cotton rats does not depend on rapid clearance.

Opsonization of Virulent VEE Virus. Table VI illustrates that when virulent (³²P) VEE strain Trinidad is inoculated into hamsters immunized

against VEE, virus is cleared from the blood and concentrated in the liver. The probable role of specific antibody is indicated by the observation that virus mixed with a 1:10 dilution of serum from hamsters immunized against VEE, is cleared when inoculated into normal, unimmunized hamsters. VEE virus mixed with antiserum raised to WEE, is not cleared.

TABLE VI. CLEARANCE AND DISTRIBUTION OF TRINIDAD STRAIN VEE IN NORMAL OR IMMUNE HAMSTERS (n=10-14)

ANTIBODY	HAMSTER	\log_{10} (^{32}P) TRIN VEE CLEARED			% IN LIVER
		1 min	5 min	30 min	
-	normal	0.07	0.10	0.13	5.9
-	VEE-immune	0.53	1.93	2.31	37.1
Anti-VEE	normal	0.18	1.23	1.93	27.3
-	WEE-immune	0.02	0.13	0.09	6.1
Anti-WEE	normal	0.00	0.03	0.00	6.0

Electron microscopic examination of (^{32}P) Trin VEE mixed with anti-VEE or anti-WEE serum revealed that the virus became aggregated when mixed with the homologous, but not with the heterologous, antiserum. This observation was complemented by the data in Table VII, which illustrate that when 1.0 ml of ^{32}P -labeled virus was mixed with its homologous antibody, a significant proportion of the ^{32}P label failed to pass a $0.45\text{-}\mu$ filter, suggesting the formation of large aggregates including virus.

TABLE VII. FORMATION OF VEE VIRUS/ANTIBODY AGGREGATES

VIRUS	ANTISERUM	CPM		
		Pre-filtrate	$0.45\text{-}\mu$ filtrate	$0.45\text{-}\mu$ filter
VEE	anti-VEE	3454	2830	6460
VEE	anti-WEE	3505	3466	1103
WEE	anti-WEE	3702	2974	5930
WEE	anti-VEE	3850	3702	1333

The removal of virus/antibody complexes by the liver may serve as an amplification system for the direct neutralization of virus by interaction with antibody. The primary effect of antibody may be only to aggregate virus (and thus reduce the infectious virus titer). The combination of antibody-initiated aggregation and subsequent removal of complexes may result in the

complete elimination of virus from the circulation of the intact animal.

Presentation:

Jahrling, P. B. Vascular clearance of alphaviruses: a correlate to virulence. Presented, Workshop on Togavirus Replication, Walter Reed Army Institute of Research, Washington, DC, 29-30 Apr 76.

Publications:

1. Jahrling, P. B., and L. Gorelkin. 1975. Selective clearance of a benign clone of Venezuelan equine encephalitis virus from hamster plasma by hepatic reticuloendothelial cells. *J. Infect. Dis.* 132:667-676.
2. Jahrling, P. B. 1975. Interference between virulent and vaccine strains of Venezuelan encephalitis virus in mixed infections of hamsters. *J. Gen. Virol.* 28:1-8.
3. Jahrling, P. B. 1976. Chromatographic separation of Venezuelan and Western encephalitis virus subtypes. *Arthropod-Borne Virus Information Exchange* 30:137-140.
4. Marker, S. C., and P. B. Jahrling. 1976. Correlation of in vitro absorption efficiency and pH-dependence with in vivo clearance and virulence of group A arboviruses. *Am. Soc. Microbiol., Abstracts of the Meeting-1976*, p. 210.
5. Jahrling, P. B., E. Navarro, and W. F. Scherer. 1976. Interferon induction and sensitivity as correlates to virulence of Venezuelan encephalitis virus for hamsters. *Arch. Virol.* 51: in press.
6. Jahrling, P. B. 1976. Virulence heterogeneity of a predominantly avirulent Western encephalitis virus population. *J. Gen. Virol.* 32: in press.

LITERATURE CITED

1. Jahrling, P. B., and W. F. Scherer. 1973. Growth curves and clearance rates of virulent and benign Venezuelan encephalitis viruses in hamsters. *Infect. Immun.* 8:456-462.
2. Jahrling, P. B., and L. Gorelkin. 1975. Selective clearance of a benign clone of Venezuelan equine encephalitis virus from hamster plasma by hepatic reticuloendothelial cells. *J. Infect. Dis.* 132:667-676.
3. Jahrling, P. B. 1976. Chromatographic separation of Venezuelan and Western encephalitis virus subtypes. *Arthropod-Borne Virus Information Exchange* 30:137-140.
4. Hilmas, D. E., W. E. Houston, R. T. Faulkner, J. R. Brown, C. L. Crabbs, and R. O. Spertzel. 1975. Vascular clearance of live, attenuated VEE virus, TC-83, in monkeys. *IRCS Med. Sci. (Immunol. Allergy)* 3:234.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION# DA OD6420	2. DATE OF SUMMARY# 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SURVEY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY ACTV# U	6. WORK SECURITY# U	7. REGIONS# NA	8. DOD/N INSTN# NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES# a. PRIMARY 62760A	PROGRAM ELEMENT PROJECT NUMBER 3A762760A834	11. TASK AREA NUMBER 01		12. WORK UNIT NUMBER 408		
b. CONTRIBUTING						
c. EQUIPMENT CARDS 114(e) (f)						
13. TITLE (Please mark Security Classification Code) (U) Pathology of Bolivian hemorrhagic fever						
14. SCIENTIFIC AND TECHNOLOGICAL AREAS# 003500 Clinical medicine; 004900 Defense; 002600 Biology (Pathology)						
15. START DATE 72 10	16. ESTIMATED COMPLETION DATE CONT	17. FUNDING AGENCY DA	18. PERFORMANCE METHOD C. In-house			
19. CONTRACT/GRAANT		20. RESOURCES ESTIMATE		21. PROFESSIONAL MAN YRS		
a. DATES/EFFECTIVE: b. NUMBER: c. TYPE: d. KIND OF AWARD:		EXPIRATION: NA	FISCAL YEAR 76	1.0	22. FUNDS IN DOLLARS 70	
		4. AMOUNT: f. CUM. AMT.	CURRENT 77	1.0	51	
23. RESPONSIBLE DOO ORGANIZATION		24. PERFORMING ORGANIZATION				
NAME# USA Medical Research Institute of Infectious Diseases ADDRESS# Fort Detrick, MD 21701		NAME# Pathology Division USAMRIID ADDRESS# Fort Detrick, MD 21701				
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (PUNIC NAME IN U.S. GOVERNMENT SPONSORED) NAME# Elwell, M. R. TELEPHONE: SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME:				
25. GENERAL USE Foreign intelligence considered		POC:DA				
26. KEY WORDS (Please mark Security Classification Code) (U) Pathology; (U) Hemorrhagic fever; (U) Machupo virus; (U) Arenaviruses; (U) Macaque monkeys; (U) BW defense; (U) Military medicine						
27. TECHNICAL OBJECTIVE# 28. APPROACH. 29. PROGRESS (Mark the appropriate numbered item(s) by number. Please mark Security Classification Code.)						
23 (U) Study the pathogenesis of Bolivian hemorrhagic fever (BHF) in the nonhuman primate and appropriate rodent models. Understanding the sequence of events occurring in BHF, their pattern of development, and effects on the host is necessary for the formulation of an effective means of treatment and protection for man. Furthermore, characterization of the disease in animal models is necessary for future evaluation of vaccines to be developed for the protection of U.S. fighting forces.						
24 (U) Perform complete necropsies on rhesus monkeys dying after inoculation with Machupo virus. Record salient gross lesions; examine tissues histologically.						
25 (U) 75 07 - 76 06 - Monkeys that survive acute BHF infection often develop a wasting neurovascular disease characterized by lymphoreticular vasculitis and perivasculitis of the central nervous system, peripheral nerves and ganglia. Similar vascular lesions and diffuse lymphocytic infiltrations are seen in many other organs. Similar lesions were seen in the central nervous system of BHF-infected, immune-serum treated rhesus monkeys that were clinically normal. In most cases it has not been possible to correlate clinical signs with degree of severity of the microscopic lesions. These unexpected findings in chronic fatal cases and in "successfully" treated monkeys raise the question as to whether BHF infection exists in humans as an unrecognized neurovascular disease. The predominance of lymphocytes in chronic BHF infection in rhesus monkeys suggests an immunological pathogenesis.						
The African green monkey was infected with BHF virus and observed to follow a course very similar to that of the rhesus; both the acute and chronic forms of disease occurred in this group. Currently a large number of long-term, clinically normal survivors of BHF infection, with and without immune serum treatment, are being necropsied and examined histopathologically.						
Publication: Am. J. Pathol. 84:211-224, 1976.						
DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE. G.U.S. GPO: 1974-240-000/000						

ENCLOSURE PAGE EIGHT NOT STAMPED

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 408: Pathology of Bolivian Hemorrhagic Fever

Background:

BHF is endemic to parts of northern Bolivia. Machupo virus, a member of the Tacaribe group of the arenaviruses, is the etiologic agent. In humans, the disease is seen as a hemorrhagic shock syndrome with high mortality. Pathology of the disease in humans has been reported by Child et al.¹ Characteristic lesions in human cases are widespread hemorrhages and congestion, hepatic necrosis, and activation of the RES with erythrophagocytosis. The rhesus monkey (*Macaca mulatta*) has been found to be an excellent model for the study of BHF, and a description of the acute infection was reported earlier.² Emphasis is presently given to chronic disease in rhesus monkeys.

Necropsies and microscopic examinations were performed on rhesus monkeys that died or were killed after inoculation with Machupo virus. Chronic disease developed most often in monkeys that were treated with immune serum after signs of illness appeared. Tremors, paresis, incoordination and muscle atrophy have been common signs in chronic BHF.

Progress:

The African Green monkey was infected with BHF virus and observed to follow a course similar to the rhesus (see also work unit 834 03 405). Both the acute and chronic forms of disease occurred in this group. Microscopically the acute form was characterized by more hemorrhage and thrombus formation in the African Green monkey than in the rhesus.

To date, all treated and untreated monkeys surviving the acute stage of infection have shown pathological changes in the central nervous system. At this time all remaining survivors of BHF challenge are being sacrificed and necropsied. Histopathological examination has not been performed on these animals at the time of this report.

Publication:

McLeod, C. G., J. L. Stookey, G. A. Eddy, and S. K. Scott. 1976.
Pathology of chronic Bolivian hemorrhagic fever in the rhesus monkey.
Am. J. Pathol. 84:211-224.

LITERATURE CITED

1. Child, P. L., R. B. MacKenzie, L. R. Valverde, and K. M. Johnson. 1967. Bolivian hemorrhagic fever. A pathologic description. Arch. Pathol. 83: 434-445.
2. Terrell, T. G., J. L. Stookey, G. A. Eddy, and M. D. Kastello. 1973. Pathology of Bolivian hemorrhagic fever in the rhesus monkey. Am. J. Pathol. 73: 477-494.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION# DA OA6427	2. DATE OF SUMMARY* 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SURVEY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY* U	6. WORK SECURITY* U	7. REGRADING* NA	8. DIA/CIO/N INSTRN# NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: a. PRIMARY 62760A	PROGRAM ELEMENT	PROJECT NUMBER 3A762760A834		TASK AREA NUMBER 01	11. LEVEL OF SUB A. WORK UNIT 801	
b. CONTRIBUTING	c. Subprograms CARDS 114(e)(f)					
12. TITLE (Provide with Security Classification Code)* (U) Radioimmunoassay techniques and their use in infectious disease research						
13. SCIENTIFIC AND TECHNOLOGICAL AREAS* 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry						
14. START DATE 73 01	15. ESTIMATED COMPLETION DATE CONT		16. FUNDING AGENCY DA	17. PERFORMANCE METHOD C. In-house		
18. CONTRACT/GRANT	19. DATES/EFFECTIVE:		20. EXPIRATION:	21. RESOURCES ESTIMATE FISCAL YEAR	22. PROFESSIONAL MAN YRS CURRENT	23. FUNDS (\$ in thousands) 193.8
				76	1.0	
				77	1.0	141.0
24. RESPONSIBLE DOG ORGANIZATION						
NAME* USA Medical Research Institute of Infectious Diseases ADDRESS* Fort Detrick, MD 21701						
25. RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833						
26. GENERAL USE Foreign intelligence considered						
27. TECHNICAL OBJECTIVE* 28. APPROACH, 29. PROGRESS (Provide individual paragraphs identified by number. Provide list of code with Security Classification Code) 23 (U) Provide rapid precise methods for measurement of biologically important compounds, with emphasis upon those which prove to be of either prognostic, diagnostic or therapeutic value during infection with agents of military significance. Currently available techniques will be exploited in the evaluation of the effectiveness of new therapeutic methods, such as hyperalimentation, while developmental efforts continue in diagnostic areas, as practical possibilities for the use of radioimmunoassay (RIA) techniques become evident. 24 (U) Development of hormone assay techniques for the measurement of glucoregulatory hormones. Monitor their alterations throughout the course of various infectious diseases in man and animals. Interpretation of these data and their relationship to host response to infection. 25 (U) 75 07 - 76 06 - Animal studies are in progress to define further the effects of LEM upon the glucoregulatory hormones. These studies indicate that some of the metabolic changes may be associated with LEM-induced alterations of the glucoregulatory hormones, which result in a significant increase in the liver second messenger system cyclic AMP, glycogenolysis and amino acid uptake. Preliminary data indicate that LEM contains prostaglandins and that they may be the active compounds in LEM. The L-dopa studies have resulted in findings which indicate that the monoamines may play an important physiologic role in the regulation of the endocrine pancreas. The data have been submitted for publication.						
Publications: N. Engl. J. Med. 293:589-591, 1975						
Available to contractors upon contractor's agreement.						
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BODY OF REPORT

Project No. 3A762760A834: Medical Defense Aspects of Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 801: Radioimmunoassay Techniques and Their Use in Infectious Disease Research

Background:

The role of the glucoregulatory hormones, insulin and glucagon, during infection are not well defined. Descriptive studies have documented the hormonal and metabolic alterations associated with infection. These changes include: hyperglucagonemia,¹ hyperinsulinemia,¹ elevated hepatic cyclic AMP concentrations,¹ glycogenolysis,¹ enhanced hepatic [¹⁴C]cycloleucine and/or [¹⁴C]α-aminoisobutyric acid (AIB) uptake, increased glucose oxidation rates,^{2,3} and decreased plasma ketone body concentrations.⁴ The observation that leukocytic endogenous mediator (LEM) results in similar metabolic and hormonal changes is new. The observation of increased plasma insulin and glucagon concentrations following LEM treatment of healthy rats has been actively pursued during the past year.

Progress:

Working on the hypothesis that the hormonal responses following LEM inoculation were responsible for some of the LEM-induced metabolic sequelae we have documented a detailed time course following inoculation of rats with LEM and single-time point conformational studies.

IP inoculation of healthy rats with LEM has the following sequential effects: a) one hour after the injection of 1 ml of LEM, both insulin and glucagon are elevated; b) by 2 hr [¹⁴C]AIB hepatic uptake is enhanced, concomitantly hepatic cyclic AMP is elevated and liver glycogen is depressed; c) peak levels of insulin, glucagon and hepatic [¹⁴C]AIB are apparent at 5 hr, hepatic glycogen is depleted and plasma glucose is suppressed; d) by 24 hr most of these parameters have returned toward control levels, whereas increased concentrations of α₂-macrofetoprotein (α₂-MFP) are apparent.

In addition to these studies, dose-response studies have been analyzed, indicating that there is a significant ($P < 0.001$) dose response for insulin, glucagon and hepatic [¹⁴C]AIB uptake following various doses of LEM. Furthermore, the hepatic [¹⁴C]AIB uptake correlates strongly ($P < 0.001$) with the I/G molar ratio. Studies in diabetic rats (Alloxan or streptozotocin induced) support the correlation between the hormones and the hepatic

[¹⁴C]AIB flux, since in both cases of chemically induced diabetes the total or partial lack of insulin either diminishes or prevents the flux of amino acid into the liver.

In situ perfused pancreas studies were performed in collaboration with Mr. Frank Beall and LTC P. Z. Sobocinski (Work Unit 834 02 111) to ascertain the action of LEM upon the pancreas, thus far these studies have proven inconclusive.

Evidence of a central nervous system effect of LEM has been obtained in collaborative studies with CPT P. T. Bailey (Work Unit 834 01 805) and LTC Sobocinski. Collaborative studies with Dr. Carol Mapes (Work Unit 834 03 011) indicate that prevention of prostaglandin synthesis with either indomethicin or aspirin during mediator (LEM) preparation results in LEM preparations that are not active for at least insulin, glucagon and amino acid flux parameters. Furthermore, addition of prostaglandins to previously inactive preparations (which have been shown not to contain prostaglandins) activates the insulin, glucagon and hepatic amino acid fluxing factors.⁵ Therefore, it appears that prostaglandins may be the active moiety contained within the LEM preparations for these parameters. If these observations are proven correct, i.e., prostaglandins are indeed the active moiety, it could also explain the direct effect of LEM upon the liver,³ since prostaglandins can increase cyclic AMP⁶ and thereby probably enhance AIB uptake.

These studies have resulted in a proposed working hypothesis explaining the role of insulin and glucagon during infection. The hypothesis is based upon the assumption that LEM is the primary effector of the hormonal and subsequent metabolic sequelae.

H₀= LEM acts upon the central nervous system initiating release of at least insulin and glucagon. Glucagon action is manifested via the second messenger system and initiates glycogenolysis and gluconeogenesis. Insulin's role appears to be at the cellular-membrane level, enhancing transport of glucose, amino acid and the other nutrients at a rate sufficient to maintain the accelerated metabolism associated with infection. Studies are in progress to define the areas within the hypothalamus which may be responsible for regulation of the insulin and glucagon responses.

In conclusion we are working on a modification of the original hypothesis explaining the metabolic actions of LEM and infection. These studies are being done in collaboration with Drs. Mapes, Bailey and Sobocinski.

Publications:

1. Rayfield, E. J., D. T. George, H. L. Eichner, and T. H. Hsu. 1975. L-dopa stimulation of glucagon secretion in man. *N. Engl. J. Med.* 293:589-591.
2. George, D. T., F. B. Abeles, M. C. Powanda, P. Z. Sobocinski, T. V. Zenser, and C. A. Mapes. 1976. Alterations in plasma insulin, glucagon, glucose, hepatic cyclic AMP and liver glycogen by a leukocyte derived factor(s). *The Endocrine Society, Program and Abstracts*, no. 491.

LITERATURE CITED

1. Curnow, R. T., E. J. Rayfield, D. T. George, T. V. Zenser, and F. R. DeRubertis. 1976. Altered hepatic glycogen metabolism and gluco-regulatory hormones during sepsis. *Am. J. Physiol.* 230: in press.
2. Long, C. L., J. L. Spencer, J. M. Kinney, and J. W. Geiger. 1971. Carbohydrate metabolism in man: effect of elective operations and major injury. *J. Appl. Physiol.* 31:110-116.
3. Cherrington, A. D., and M. Vranic. 1974. Effect of interaction between insulin and glucagon on glucose turnover and FFA concentration in normal and depancreatized dogs. *Metabolism* 23:729-744.
4. Neufeld, H. A., J. A. Pace, and F. E. White. 1976. The effect of bacterial infections on ketone concentrations in rat liver and blood and on free fatty acid concentrations in rat blood. *Metabolism* in press.
5. Mapes, C. A., D. T. George, and P. Z. Sobocinski. 1976. Possible interrelationships of prostaglandins and PMN-derived mediators of the host inflammatory response. *Prostaglandins*, submitted.
6. Zenser, T. V., F. R. DeRubertis, and R. T. Curnow. 1974. Effects of prostaglandins on hepatic adenylate cyclase activity and cyclic adenosine 3',5'-monophosphate content. *Endocrinology* 94:1404-1410.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ²		2. DATE OF SUMMARY ³		REPORT CONTROL SYMBOL							
3. DATE PREV SURVY ⁴ 75 07 01		4. KIND OF SUMMARY D. CHANGE		5. SUMMARY SCTY ⁵ U		6. WORK SECURITY ⁶ U		7. REGARING ⁷ NA		8. ORIGIN INSTN ⁸ NL		9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO		10. LEVEL OF SUM A. WORK UNIT	
10. NO./CODES: ⁹		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER				WORK UNIT NUMBER					
a. PRIMARY		62760A		3A762760A834		01				802					
b. CONTRIBUTING															
c. Information		CARDS 114(e)(f)													
11. TITLE (Provide with Security Classification Code) (U) Microbial toxins and their role in the pathogenesis of disease															
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ¹⁰ 003500 Clinical medicine; 004900 Defense; 002600 Biology (Pathology)															
13. START DATE 66 10		14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-house									
17. CONTRACT/GRAANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS 1.0		20. FUNDS (in thousands) 84.0									
a. DATES/EFFECTIVE:		EXPIRATION:		FISCAL YEAR		76									
b. NUMBER: ¹¹ NA		4. AMOUNT:		CURRENT		77									
c. TYPE:		5. CUM. AMT.													
d. KIND OF AWARD:															
19. RESPONSIBLE DOO ORGANIZATION		NAME: ¹² USA Medical Research Institute of Infectious Diseases		NAME: ¹³ Pathology Division		ADDRESS: ¹² Fort Detrick, MD 21701		NAME: ¹³ USAMRIID		ADDRESS: ¹³ Fort Detrick, MD 21701					
		TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (PUNISH DEAN // U.S. Academic Institution) NAME: ¹⁴ Metzger, J. F.				TELEPHONE: 301 663-7211		SOCIAL SECURITY ACCOUNT NUMBER:					
21. GENERAL USE		22. ASSOCIATE INVESTIGATORS		NAME: ¹⁴ Johnson, A. D.		NAME: ¹⁴								POC:DA	
23. APPROACH, PROGRESS (Provide individual paragraphs identified by number. Provide each with Security Classification Code) (U) Enterotoxin; (U) Staphylococcus; (U) Escherichia coli; (U) Military medicine; (U) Isotopic tracers; (U) Antigen; (U) Antibody; BW defense															
24. TECHNICAL OBJECTIVE, APPROACH, PROGRESS (Provide individual paragraphs identified by number. Provide each with Security Classification Code) 23 (U) Study production and purification and characterize microbial toxins. Use the purified toxins to produce toxoids which can then be tested for safety and efficacy as new immunizing agents. This work unit is an essential element in a comprehensive program for medical defense against BW agents, because it is aimed at developing and testing new toxoids for use in military forces.															
24 (U) Purify toxins of microbial origin in order to study the pharmacologic effects. Determine the efficacy of an oral immunization to E. coli enterotoxin.															
25 (U) 75 07 - 76 06 - Development of a hemagglutination test for exfoliative toxins was accomplished. A formalin toxoid was prepared of exfoliatin. Investigations were conducted to determine the molecular weight of the active toxin moiety of E. coli enterotoxin.															
Publications: Infect. Immunity 12:93-97, 1206-1210, 1975. J. Immunol. 115:49-53, 1975.															
Available to contractors upon contractor's approval.															

DD FORM 1 MAR 68 1498

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AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

* U.S. GPO: 1974-840-843/8691

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 802: Microbial Toxins and Their Role in the Pathogenesis of Disease

Background:

Certain strains of Escherichia coli have been isolated which produce an enterotoxin similar to the toxin produced by Vibrio cholera.^{1,2} Methods for cultivation of E. coli that produce optimum amounts of toxin have been reported previously.^{2,3} To date, the main emphasis of research has been directed toward establishing assay procedures for the E. coli toxin, and toward purification steps to isolate the toxin. Several assay procedures have been published^{2,4,6,7} and each has its advantages and disadvantages. Attempts at purification have been reported, but there has not been a significant amount of pure material produced by any group for further study.^{4,5}

Staphylococcal exfoliatin is responsible for scalded-skin syndrome in children. The toxin responsible has been isolated and characterized by several independent groups of investigators.^{8,9} We have begun to look at toxoiding and other immunological parameters of the exfoliatin.

Progress:

E. coli enterotoxin as well as most bacterial exoproteins is produced primarily in the late log to early stationary phase of bacterial growth. Utilizing control conditions of fermentation, replicate lots have uniform toxicity in either a rabbit loop assay or in the adrenal cell assay. Prolonged fermentation beyond 24 hr does not increase the toxicity. Ultrafiltration studies utilizing Amicon membranes reveal no heat-labile toxicity at a MW of < 50,000. Over 95% of the toxicity is retained by an XM-100A membrane, indicating that major toxin concentration has a MW of > 100,000. The crude concentrated material was chromatographed on Sepharose 2B, 4B, 6B, Sephadex G-200 and G-100. The major toxic activity was in the void volume from all these chromatographic procedures. There was a small fraction which represented 1% of the original activity which had a MW of 60,000. This low MW toxic material has essentially the same specific activity on a protein basis as the original crude culture supernatant. Dorner,⁴ Finklestein et al.,² and Evans et al.,³ have partially purified similar low MW substances having toxic activity, with no appreciable increase in specific activity over the crude toxin preparation. These findings suggest that the major E. coli enterotoxin

is tightly bound to high MW capsular polysaccharide and/or lipopolysaccharide. Crude preparations having high specific activity are only 30% protein, the rest being lipids and sugars.

Because these toxic low MW fractions are negligible in quantity, preliminary formalin-toxoiding procedures of crude culture supernatant have been accomplished. Toxic activity is reduced by a factor of 5 logs after 30-day toxoiding at 37°C as determined by the adrenal cell rounding assay. Toxoided material and crude toxin suspended in alkaline emulsion will be administered by stomach tube to rabbits in an attempt at oral immunization. Animals will be challenged with both *V. cholerae* and *E. coli* toxin utilizing the rabbit loop technique.

Several lots of pure exfoliatin were prepared as previously described. All lots were checked for homogeneity by SDS gel electrophoresis, and for biologic activity using the newborn mouse model.

One hundred milligrams of pure toxin were subjected to 0.1%-formalin toxoiding at 37°C for 28 days. Samples were removed weekly, dialyzed to remove formalin, and examined for changes in molecular size and/or remaining biologic activity. After one week, there was no activity remaining, as determined by injecting mice with 5 times the median dose. By SDS electrophoresis, the formalinized product had polymerized into large molecules, representing dimers, trimers, and polymers of the original molecule.

Both the toxoided material described here, and the pure toxin were used to immunize rabbits. Weekly injections of 1 mg were used for 2 weeks followed by 4 injections of 1.5 mg. Trial bleedings during the course of immunization were studied for precipitin reactions and for HA titers. The results are summarized in Table I.

TABLE I. IMMUNIZATION REACTIONS TO EXFOLIATIN TOXIN AND TOXOID

RABBIT NO.	ANTIGEN	WEEKS AFTER IMMUNIZATION	TITER	
			PRECIPITIN	HA
139	Toxoid	0	0	0
		6	None	1:2560
140	Toxoid	0	0	0
		6	None	1:320
141	Toxin	0	0	0
		6	1:8	1:80,000
142	Toxin	0	0	0
		6	1:4	1:40,000

Using this immune serum a radial immunodiffusion assay was established to: (1) quantitate toxin production by bacterial strains, and (2) follow yield of toxin during purification procedures. Antiserum is diluted 1:50 in cooled 1.5% Ionagar, glass slides are poured, and the agar is allowed to harden. Wells, 4 mm in diameter, are punched in the agar, and 10- μ l samples of reference toxin or unknown solutions are pipetted into the wells. By measuring the diameter of the zone of precipitation around each well, standard curves can be constructed, and toxin values of the unknown determined from this curve. Concentrations of < 0.5 μ g/ml can be determined by this method.

Publications:

1. Metzger, J. F., A. D. Johnson, and L. Spero. 1975. Intrinsic and chemically produced microheterogeneity of Staphylococcus aureus enterotoxin type C. *Infect. Immun.* 12:93-97.
2. Warren, J. R., D. L. Leatherman, and J. F. Metzger. 1975. Evidence of cell-receptor activity in lymphocyte stimulation by staphylococcal enterotoxin. *J. Immunol.* 115:49-53.
3. Johnson, A. D., J. F. Metzger, and L. Spero. 1975. Production purification, and chemical characterization of Staphylococcus aureus exfoliative toxin. *Infect. Immun.* 12:1206-1210.

LITERATURE CITED

1. Gyles, C. L. 1974. Relationships among heat-labile enterotoxins of Escherichia coli and Vibrio cholerae. *J. Infect. Dis.* 129:277-283.
2. Finkelstein, R. A., and K. Punyashthiti. 1974. Studies on Escherichia coli enterotoxin(s). p. 305-323. In Proceedings, 9th Joint Cholera Research Conference, Grand Canyon, Arizona, 1-3 October 1973, Department of State Publication 81762, Bureau of International Scientific and Technological Affairs, Washington, DC.
3. Evans, Jr., D. J., D. G. Evans, and S. L. Gorbach. 1974. Polymyxin B-induced release of low-molecular-weight, heat-labile enterotoxin from Escherichia coli. *Infect. Immun.* 10:1010-1017.
4. Dorner, F. 1975. Escherichia coli enterotoxin. Purification and partial characterization. *J. Biol. Chem.* 250:8712-8719.
5. Möllby, R., S. G. Hjalmarsson, and T. Wadström. 1975. Separation of E. coli heat-labile enterotoxin by preparative isotachophoresis. *FEBS Letters* 56:30-33.

6. Gill, D., and C. A. King. 1975. The mechanism of action of cholera toxin in pigeon erythrocyte lysates. *J. Biol. Chem.* 250:6424-6432.
7. Kwan, C. N., and R. M. Wishnow. 1974. Escherichia coli enterotoxin-induced steroidogenesis in cultured adrenal tumor cells. *Infect. Immun.* 10:146-151.
8. Melish, M. E., and L. A. Glasgow. 1970. The staphylococcal scalded-skin syndrome. Development of an experimental model. *N. Engl. J. Med.* 282:1114-1119.
9. Kapral, F. A., and M. M. Miller. 1971. Product of Staphylococcus aureus responsible for the scalded-skin syndrome. *Infect. Immun.* 4:541-545.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^b DA OA6429	2. DATE OF SUMMARY ^b 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SURVEY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SECY ^a U	6. WORK SECURITY ^a U	7. REGRADING ^b NA	8. DR&E'S INSTR'N NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM- A WORK UNIT
10. NO./CODES: ^a PROGRAM ELEMENT PROJECT NUMBER				TASK AREA NUMBER		WORK UNIT NUMBER	
B. PRIMARY 62760A	3A762760A834			01	803		
C. CONTRIBUTING							
C. CONFIDENTIAL CARDS 114(e)(f)							
11. TITLE (Proceed with Security Classification Code) (U) Subcellular biological effects of microbial disease and toxins							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a 003500 Clinical medicine; 004900 Defense; 016800 Toxicology; 002300 Biochemistry							
13. START DATE 60 07	14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-house		
17. CONTRACT/GRAANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS		20. FUNDS (In thousands)	
B. DATES/EFFECTIVE:		PRECEDING FISCAL YEAR 76		1.0		217.3	
D. NUMBER: NA		CURRENT 77		1.0		248.0	
C. TYPE:		4. AMOUNT: F. CUM. AMT.					
G. KIND OF AWARD:							
19. RESPONSIBLE DOD ORGANIZATION NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701				20. PERFORMING ORGANIZATION NAME: Bacteriology Division USAMRIID ADDRESS: Fort Detrick, MD 21701			
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833				PRINCIPAL INVESTIGATOR (Provide name // U.S. Academic Institution) NAME: Canonico, P. G. TELEPHONE: 301 663-7341 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME:			
21. GENERAL USE Foreign intelligence considered				POC:DA			
22. KEYWORDS (Proceed each with Security Classification Code) (U) Peroxisomes; (U) Hepatocytes; (U) Streptococcus pneumoniae; (U) Fatty acids; (U) Inflammation; (U) Intermediary metabolism; (U) Military medicine; (U) BW defense							
23. TECHNICAL OBJECTIVE ^a ; 24. APPROACH; 25. PROGRESS (Indicate individual paragraphs identified by number. Proceed rest of each with Security Classification Code.) 23 (U) Study the effects of infection and/or intoxication upon distribution and integrity of subcellular organelles and determine the role of tissue enzyme changes in infectious disease. This investigation will lead to information regarding the pathogenesis and treatment of infections of military importance. 24 (U) A variety of techniques, e.g., tissue fractionation, enzyme analyses, centrifugation analysis, are used to study subcellular effects of microorganisms and toxins. 25 (U) 75 07 - 76 06 - A turpentine-induced inflammatory lesion has been shown to cause a depression of 5 hepatic peroxisomal enzymes. Catalase activity, showing the greatest depression, was decreased by 60% as a result of a reduced rate of de novo synthesis. Liver cells isolated from turpentine-treated rats showed an enhanced capacity to incorporate added fatty acids into cellular lipids; the capacity to oxidize fatty acids to carbon dioxide, however, was significantly reduced. It is hypothesized that depression of peroxisomal enzyme activity in turpentine-treated rats is a normal hepatic response to inflammation and may result in inactivation of peroxisomal-mediated oxidation of cytoplasmic NADH. Glucose formation from pyruvate or lactate is depressed in isolated rat hepatocytes obtained during the aortal stages of S. pneumoniae infection. Studies using added ethanol as a source of reducing equivalents (H ion) indicate that inhibition of gluconeogenesis is not the result of a lack of H ion, but may be rather due to its impaired transport from the cytosol to the inner compartment of the mitochondria. Publications: Lab. Invest. 33:147-150, 1975. Infect. Immun. 12:42-47, 1975. Proc. Soc. Exp. Biol. Med. 149:1019-1022, 1975. Am. J. Clin. Nutr., in press, 1976.							
Available to contractors upon contractor's approval.							

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* U.S. GPO: 1974-840-843/8691

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Aspects of Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 803: Subcellular Biological Effects of Microbial Disease and Toxins

Background:

Maintaining the integrity of subcellular organelles, such as lysosomes, mitochondria, peroxisomes and endoplasmic reticulum, is essential for normal cellular functions. Alterations in the normal physiology of these organelles, can contribute to altered enzymatic and metabolic events, lead to cellular dysfunction and ultimately enhance host susceptibility to infectious diseases.¹ Characterization of the morphology and function of subcellular organelles in normal and diseased animals is, therefore, essential for delineating host defense mechanisms to infection, intoxication and inflammation, differentiating between specific and nonspecific host responses and clarifying mechanisms of bacteria- or toxin-induced pathophysiology.

Progress:

I. The name peroxisome designates a special type of intracellular respiratory particle characterized by the association of one or more H₂O₂-generating oxidases with large amounts of catalase. Hepatic peroxisomes are approximately 0.5 μ in diameter; they are bounded by a single membrane limiting a granular matrix wherein catalase and other peroxidative enzymes are located. These enzymes account for only about 1/2 of the peroxisomal proteins. The nature of the remaining proteins is still unknown, and is one of the chief reasons why the physiologic role of hepatic peroxisomes is still poorly understood. One approach for ascertaining peroxisomal function is to identify factors that are capable of altering their biochemical and structural morphology. To date only 3 such factors are known: (1) anti-hyperlipemic drugs, such as clofibrate, which cause marked proliferation of hepatic peroxisomes, (2) Zellwenger's syndrome, a rare familial disease in which peroxisomes are absent from liver, kidney and nerve tissue, and (3) bacterial infections which cause a depletion of hepatic peroxisomes.²

To determine whether a sterile inflammatory reaction could, by itself, induce a depression of peroxisomal enzyme activity, experiments were performed on rats following a SC injection of turpentine. Shown in Table I are results for certain parameters which, in part, serve to characterize the turpentine-induced sterile inflammation model. Fever occurred only on day 1. Characteristics of an inflammatory response are annotated by leukopenia on days 1-3, depression of serum Fe on day 1, and serum Zn on days 1-3; as

TABLE I. PHYSIOLOGIC AND METABOLIC RESPONSES IN RATS FOLLOWING SC INJECTION OF TURPENTINE.

PARAMETER	MEAN \pm SE BY DAYS POSTTURPENTINE INJECTION ^a				
	0	1	2	3	5
Body temperature °C	37.2 \pm 0.1	38.9 \pm 0.3 ***	37.5 \pm 0.1	37.0 \pm 0.1	37.8 \pm 0.2
WBC ($\times 10^{-3}/\text{mm}^3$)	6.22 \pm 0.48	4.00 \pm 0.29 **	4.35 \pm 0.49 *	3.94 \pm 0.29 **	6.66 \pm 0.75
FAN (%)	16.8 \pm 2.6	14.0 \pm 2.8	4.3 \pm 2.0 **	17.2 \pm 5.0	29.2 \pm 5.5 *
Serum Fe ($\mu\text{g/dl}$)	315 \pm 17	151 \pm 13 ****	328 \pm 9	315 \pm 14	376 \pm 18 *
Serum Zn ($\mu\text{g/dl}$)	125 \pm 6	86 \pm 4 ****	86 \pm 4 ***	74 \pm 7 ***	113 \pm 17
Serum α_2 -MFP (mg/ml)	1.1 \pm 0.3	35.8 \pm 7.8 ****	62.6 \pm 14.3 ***	26.2 \pm 6.3 ***	14.0 \pm 4.6 ***
Serum lysozyme ($\mu\text{g/ml}$)	2.5 \pm 0.3	3.3 \pm 0.1	4.1 \pm 0.2 **	3.7 \pm 0.2	4.3 \pm 0.2 ***
Serum β -GLUC (U/dl)	57.5 \pm 6.4	86.8 \pm 10.8 *	60.1 \pm 4.2	37.7 \pm 3.5	53.3 \pm 5.7

^a N = 10 for day 0, N = 5 for days 1-5.

* P < 0.05

** P < 0.01

*** P < 0.001

well as by the appearance of acute-phase serum proteins as depicted here by α_1 -macrofetoprotein (α_2 -MFP). Peak serum macrofetoprotein concentration occurred at 48 hr and was substantially reduced by 72 hr. Increasing levels of serum lysozyme were consistent with development of an inflammatory lesion. Serum β -glucuronidase (β -GLUC), an enzyme proposed to be an indicator of hepatic cellular damage showed only a small statistically insignificant rise on day 1.

The activity of hepatic peroxisomal enzymes following turpentine injection are shown in Table II. Catalase, urate oxidase, amino acid oxidase, and hydroxy-acid oxidase all manifested decreased activity. The greatest depression, 60% decrease by day 3, occurred with catalase. The colorimetric assay procedure employed for carnitine acyl-transferase was not sensitive enough to accurately measure the activity of this enzyme in crude liver homogenates. Hence, a 2nd group of rats was fed a diet containing 0.1% Nafenopin, an anti-hyperlipemic drug which causes a marked proliferation of hepatic peroxisomes. Its effects upon physiologic and metabolic responses and hepatic peroxisomal evidence activity are shown in Tables III and IV, respectively. Nafenopin treatment doubled the level of catalase activity and produced a 100-fold increase in carnitine acyl-transferase (Table IV). When Nafenopin-fed rats were injected with turpentine a decrease in carnitine acyl-transferase was observed, which was quantitatively and temporally similar to that observed for catalase. Of interest was the observation that Nafenopin decreases the activity of the other peroxisomal enzymes. The failure of turpentine to decrease their activity further, suggests that there is a limit to which the activity of these enzymes is lowered.

In order to assess whether an increased rate of turnover or a decrease in synthesis of enzymes or both was responsible for the observed peroxisomal enzyme depletion, the rate of catalase synthesis and degradation was determined. In this study rats were treated with a single IP injection of amino-triazole, a compound which forms an irreversible complex with catalase and reduces its activity by more than 95%. Following a delay of several hours, during which excess amino triazole is excreted, catalase activity increased as a result of de novo synthesis. The return of catalase activity toward its normal level was then determined over an 86-hr period. When amino triazole-treated rats were also injected with turpentine the return of catalase activity was significantly delayed. Since these curves follow first order kinetics, the data plotted as a log function yields a straight line, from which the rate of catalase synthesis can be calculated. The results of these calculations indicated that the rate of synthesis in control rats was equivalent to 22.6 IU/hr. In turpentine-treated rats 2 distinct rates were evident. During the first 65 hr following the administration of turpentine the rate of synthesis was reduced to 5.5 U, or 1/4 the rate calculated for control rats. Subsequent to this initial period the rate became equivalent to that of the untreated control group.

TABLE II. HEPATIC PEROXISOMAL ENZYME ACTIVITIES FOLLOWING A SC INJECTION OF TURPENTINE.

ENZYME	MEAN IU ± SE BY DAYS POSTTURPENTINE INJECTION ^a				
	0	1	2	3	5
Catalase	344 ± 12	263 ± 22 **	173 ± 20 ***	138 ± 4 ***	289 ± 1 **
Urate oxidase	4.41 ± 0.29	4.12 ± 0.39	3.36 ± 0.57	2.89 ± 0.36 *	5.03 ± 0.22
D-amino acid oxidase	7.61 ± 0.76	6.41 ± 0.28	4.56 ± 0.34 *	5.07 ± 0.21 *	4.78 ± 0.53 *
Hydroxy-acid oxidase	12.4 ± 0.92	10.2 ± 0.69	6.79 ± 0.13 **	6.67 ± 0.17 ***	6.77 ± 0.39 ***
Carnitine acyl-transferase	0.55 ± 0.28	0.43 ± 0.17	0.30 ± 0.06	0.91 ± 0.32	0.53 ± 0.16

^a N = 10 for day 0, N = 5 for days 1-5.

* P < 0.05

** P < 0.01

*** P < 0.001

TABLE III. PHYSIOLOGIC AND METABOLIC RESPONSES IN NAFENOPIN-FED RATS FOLLOWING A SC INJECTION OF TURPENTINE.

PARAMETER	MEAN \pm SE BY DAYS POSTTURPENTINE INJECTION ^a				
	0	1	2	3	5
Body temperature °C	37.1 \pm 0.2	39.5 \pm 0.2 ***	38.1 \pm 0.2 **	37.5 \pm 1	37.7 \pm 0.2
WBC ($\times 10^{-3}$ /mm ³)	6.94 \pm 0.47	3.52 \pm 0.31 ***	3.92 \pm 0.23 ***	6.14 \pm 0.64	6.20 \pm 0.66
PMN (%)	16.2 \pm 3.1	12.0 \pm 2.7	3.1 \pm 0.8 **	12.0 \pm 0.6	16.1 \pm 1.6
Serum iron ($\mu\text{g}/\text{dl}$)	314 \pm 12	159 \pm 18 ***	255 \pm 18 *	227 \pm 16	302 \pm 17
Serum zinc ($\mu\text{g}/\text{dl}$)	116 \pm 3	70 \pm 2 ***	89 \pm 2 ***	89 \pm 4 ***	91 \pm 3 ***
Serum O ₂ -HbP (mg/ml)	0.5 \pm 0.3	13.0 \pm 1.8 ***	22.6 \pm 5.3 ***	25.2 \pm 4.1 ***	8.0 \pm 3.0 **
Serum lysozyme ($\mu\text{g}/\text{ml}$)	2.5 \pm 0.1	3.2 \pm 0.1 ***	4.2 \pm 0.3 ***	4.4 \pm 0.3 ***	4.0 \pm 0.2 ***
Serum β -GLUC (U/dl)	72.7 \pm 7.1	115.8 \pm 8.5 ***	107.2 \pm 4.7 **	73.0 \pm 7	75.0 \pm 12.3

a N = 10 for day 0, N = 5 for days 1-5.

* P < 0.05

** P < 0.01

*** P < 0.001

TABLE IV. HEPATIC PEROXISOMAL ENZYME ACTIVITIES IN NAFENOPIN-FED RATS FOLLOWING A SC INJECTION OF TURPENTINE.

ENZYME	MEAN IU ± SE BY DAYS POSTTURPENTINE INJECTION ^a				
	0	1	2	3	5
Catalase	681 ± 28	487 ± 34 **	429 ± 46 ***	376 ± 15 ***	502 ± 51 ***
Urate oxidase	3.10 ± 0.30	3.28 ± 0.44	3.31 ± 0.17	3.22 ± 0.18	3.42 ± 0.31
D-amino acid oxidase	1.24 ± 0.08	0.96 ± 0.05	0.77 ± 0.09	0.81 ± 0.03 **	1.09 ± 0.23
Hydroxy-acid oxidase	3.76 ± 0.45	2.80 ± 0.28	2.55 ± 0.22	3.01 ± 0.10	3.72 ± 0.45
Carnitine acyl-transferase	60.9 ± 5.3	42.7 ± 4.4	35.0 ± 2.3 **	37.3 ± 9.3 *	41.5 ± 4.4 *

^a N = 10 for day 0, N = 5 for days 1-5.

* P < 0.05

** P < 0.01

*** P < 0.001

It should be noted that the change in the rate of catalase synthesis occurs at approximately the same time as the change in the concentration of the acute phase serum protein α_2 -MFP. This observation supports an earlier suggestion that the synthesis of acute-phase serum proteins may occur at the expense of peroxisomal protein synthesis and results in a reduction of the peroxisomal protein pool and, hence, in the number of peroxisomes. Consistent with this hypothesis is the observation that the rate of catalase synthesis in kidney was not altered by turpentine. Catalase synthetic rate in kidney of turpentine-treated rats was 4.3 IU/hr, a value which is essentially identical to the 4.9 IU/hr obtained for untreated rats.

The relationship of these observations to liver function during sepsis is not clearly understood. However, a review of the known relationships between peroxisomes and cellular functions implicates a possible participation by peroxisomes in lipid and carbohydrate metabolism.

For this reason certain aspects of lipid and carbohydrate metabolism in liver cells was examined. The suspensions of freshly isolated hepatocytes used were obtained by *in situ* perfusion of collagenase. They demonstrate numerous metabolic functions characteristic of liver *in vivo*.³

Studies to date have employed hepatocytes isolated 48 hr after the administration of turpentine and incubated in the presence of radiolabeled fatty acids. As shown in Table V, hepatocytes from turpentine-treated rats

TABLE V. METABOLISM OF FATTY ACIDS BY ISOLATED HEPATOCYTES FROM CONTROL AND TURPENTINE-TREATED RATS.

FATTY ACID	Metabolic parameter	nmol SUBSTRATE CONVERTED/HR/5 $\times 10^6$ CELLS \pm SE (N)	
		Control rats	Turpentine-treated rats
Oleate		7.31 \pm 0.41 (9)	3.74 \pm 0.29 (9)*
Palmitate	$^{14}\text{CO}_2$ Production	6.13 \pm 1.09 (9)	3.53 \pm 0.28 (9)*
Octanoate		2.06 \pm 0.19 (9)	1.61 \pm 0.70 (9)*
Oleate		11.24 \pm 1.00 (21)	15.65 \pm 1.40 (18)**
Palmitate	Esterification into cell lipid	4.52 \pm 0.31 (21)	6.73 \pm 0.37 (18)*
Octanoate		0.80 \pm 0.06 (18)	1.31 \pm 0.05 (21)*

* P < 0.01

** P < 0.025

oxidized the added fatty acids (FA), oleate, palmitate and octanoate, as determined by the release of $^{14}\text{CO}_2$, at a significantly lower rate than control hepatocytes. On the other hand, esterification of the added FA into cellular lipid was significantly increased when compared to esterification by control cells.

When glucose formation from various precursors was measured, hepatocytes from turpentine-treated rats showed enhanced gluconeogenesis from lactate, pyruvate and alanine when 1 mM oleate was also added. If hepatic gluconeogenesis can be considered to be a common denominator in acute stages of infection or inflammation, then a physiologic explanation for depletion of hepatic peroxisomal activity might be derived from the following hypothesis.⁴

Several products of the oxidations catalyzed by peroxisomal enzymes (acetaldehyde and ketoacids) can be reduced again by dehydrogenases occurring in the cell sap. Consequently, peroxisomes may play a role in the reoxidation of extramitochondrial NADH to NAD, which would have the effect of stimulating glycolysis. During gluconeogenesis the reverse would be true and the liver cell would benefit from inactivation of this peroxisomal function since NADH not NAD is required for synthesis of glucose.

II. Conflicting reports have appeared in the literature concerning the gluconeogenic capacity of the liver during sepsis and trauma. Therefore, studies using suspensions of isolated hepatocytes were initiated in order to determine the rates of glucose formation by the addition of precursors to liver cells obtained from rats during various stages of a pneumococcal infection. Gluconeogenesis was measured in isolated fresh suspensions of hepatocytes from 40-hr fasted control rats and 40-hr fasted experimental rats inoculated 24 or 40 hr previously with 10^5 S. pneumoniae subcutaneously.

The rate of glucose formation under various incubation conditions is shown in Table VI. Glucose production from pyruvate was linear with time following an initial 15 min preincubation period, and was of similar magnitude in both fasted controls and 24-hr infected livers. However, by 40 hr there was a significant decrease in the rate of glucose production from pyruvate. It can be seen that addition of reducing equivalents in the form of ethanol (ETOH) stimulated glucose production. However, the degree of stimulation is significantly less in infected rats as compared to fasted controls. Gluconeogenesis from lactate was also reduced in hepatocytes from agonal rats and the addition of ETOH caused an even greater inhibition of glucose production than was observed in fasted controls.

These data indicate that in septic rats there is progressively increasing inhibition of gluconeogenesis. This inhibition is not the result of insufficient reducing equivalents; rather the data are consistent with the proposal that in hepatocytes from infected rats reducing equivalents in the form of NADH are not as effectively transported from the cytoplasm to the inner compartment of the mitochondria (its membrane is impermeable to NAD/NADH).

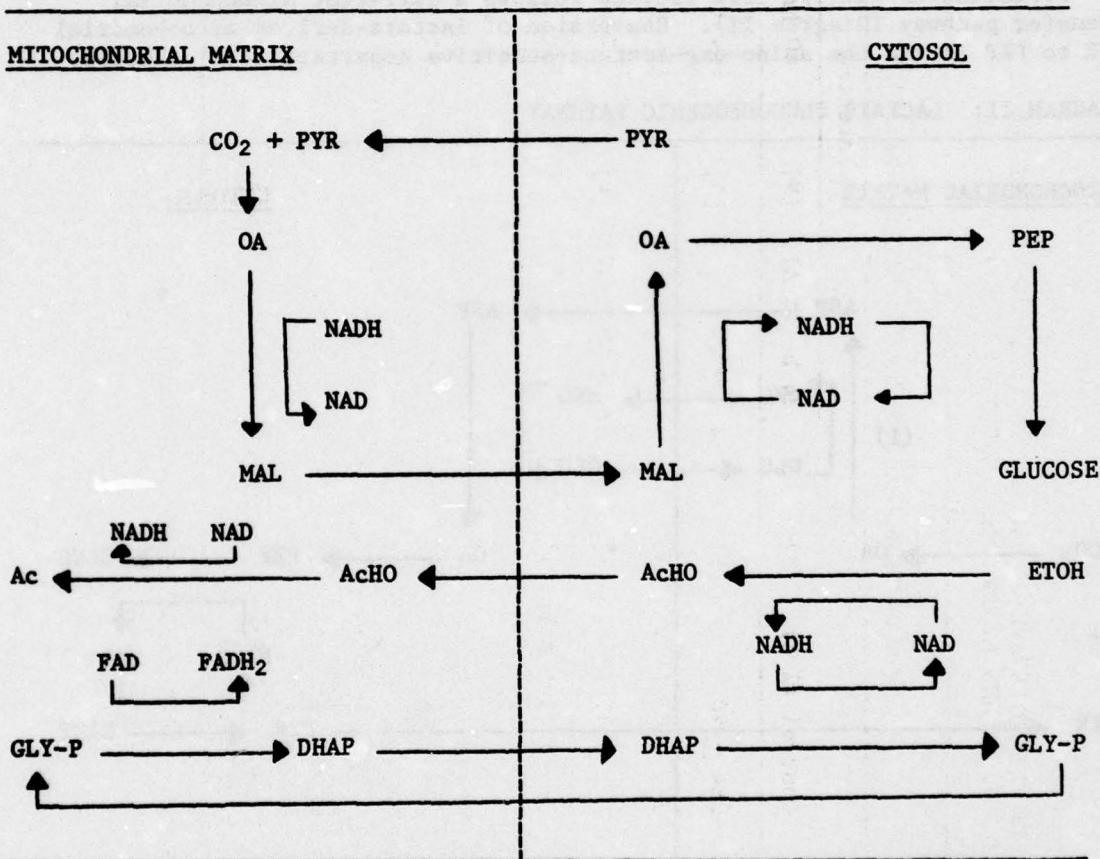
TABLE VI. GLUCONEOGENESIS FROM PYRUVATE OR LACTATE IN ISOLATED SUSPENSIONS OF HEPATOCYTES OBTAINED FROM CONTROL AND PNEUMOCOCCAL INFECTED RATS.

ASSAY CONDITIONS ^a	μg GLUCOSE FORMED/45 MIN/10 ⁷ CELLS (MEAN ± SE)					
	Infected					
	Control (Z Δ)	[N]	24 hr (Z Δ)	[N]	40 hr (Z Δ)	[N]
Pyruvate (10 mM)	284 ± 40	[5]	254 ± 22	[4]	164 ± 22	[6]
Pyruvate + AOA (0.2 mM)	295 ± 45 (+ 4)	[3]	--		195 ± 27 (+ 9)	[4]
Pyruvate + EtOH (2 mM)	528 ± 45 (+86)	[5]	373 ± 28 (+47)	[4]	237 ± 32 (+45)	[6]
Pyruvate + EtOH + AOA	465 ± 85 (+64)	[3]	--		247 ± 22 (+50)	[3]
Lactate (10 mM)	264 ± 12	[8]	--		171 ± 14	[5]
Lactate + AOA (0.2 mM)	< 5 (-98)	[2]	--		< 5 (-97)	[2]
Lactate + EtOH (2 mM)	182 ± 18 (-31)	[6]	--		73 ± 19 (-58)	[5]
Lactate + EtOH + AOA	< 5 (-98)	[2]	--		< 5 (-97)	[2]

a AOA = Amino-oxy-acetate. 2 mM NH₄Cl added to lactate.

It thus appears that the transport mechanism responsible for such transfers (i.e., α -glycerolphosphate shuttle) is functionally impaired. Formation of glucose from pyruvate requires mitochondrial NADH to convert mitochondrial oxaloacetate (OA) to malate (MAL) (Diagram I).

DIAGRAM I: PYRUVATE GLUCONEOGENIC PATHWAY



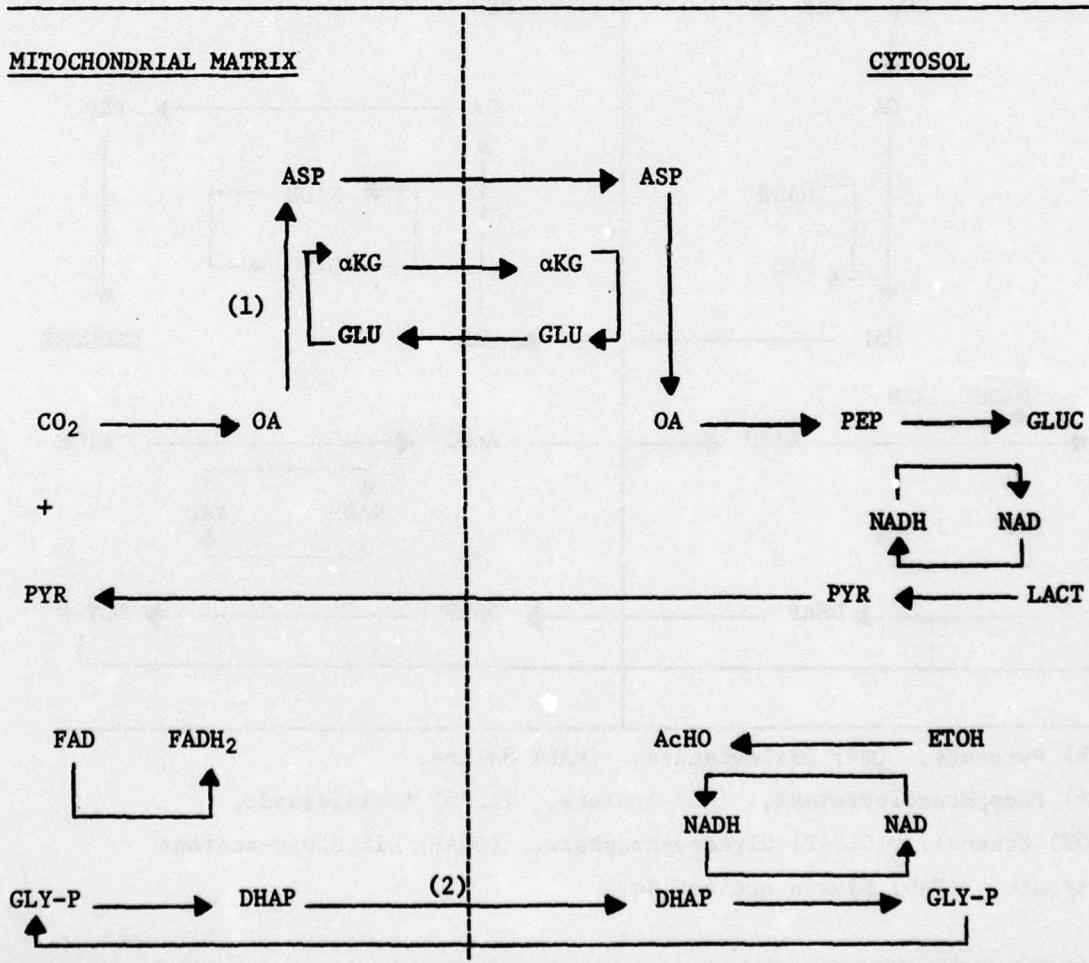
(PYR) Pyruvate, (OA) Oxaloacetate, (MAL) Malate,
 (PEP) Phosphoenolpyruvate, (Ac) Acetate, (AcHO) Acetaldehyde,
 (ETOH) Ethanol, (GLY-P) Glycerophosphate, (DHAP) Dihydroxy-acetone phosphate, (FAD) Flavin nucleotide.

Oxidation of ETOH results in increased cytoplasmic NADH which when transported to the mitochondria stimulates the conversion of OA to MAL. Malate diffuses to the cytoplasm where it is converted back to OA and then to phosphoenolpyruvate (PEP) and ultimately to glucose. In infected

rats, cytoplasmic NADH increases as a result of ETOH oxidation, and a reduction in the transport of reducing equivalents to the mitochondria results in (1) an impaired conversion of cytoplasmic MAL to OA as a result of competition for available NAD and (2) impaired conversion of mitochondrial OA to MAL due to insufficient H^+ transport.

Formation of glucose from lactate follows a different mitochondrial transfer pathway (Diagram II). Conversion of lactate-derived mitochondrial PYR to PEP is via the amino-oxy-acetate-sensitive aspartate (ASP) shuttle.

DIAGRAM II: LACTATE GLUCONEOGENIC PATHWAY



(ASP) Aspartate, (α KG) α -ketoglutarate, (GLU) Glutamate, (LACT) Lactate.

Other symbols as in Diagram I.

1 = site of amino-oxy-acetate inhibition.

2 = site of proposed inhibition of hydrogen transport in hepatocyte of septic rats.

In contrast to the findings with PYR the addition of ETOH to lactate, inhibited glucose formation because ETOH oxidation competes with the oxidation of lactate to PYR for available cytoplasmic NAD. In infected rat hepatocytes, the decreased rate of NADH transport to the mitochondria probably through the α -glycerophosphate shuttle results in maintenance of a high NADH/NAD ratio and in an enhanced inhibition of lactate conversion to pyruvate. Studies are in progress to improve methods for the direct measurement of the functional status of mitochondrial shuttles.

Publications:

1. Canonico, P. G., J. D. White, and M. C. Powanda. 1975. Peroxisome depletion in rat liver during pneumococcal sepsis. *Lab. Invest.* 33:147-150.
2. Canonico, P. G., M. C. Powanda, G. L. Cockerell, and J. B. Moe. 1975. Relationship of serum β -glucuronidase and lysozyme to pathogenesis of tularemia in immune and nonimmune rats. *Infect. Immun.* 12:42-47.
3. Ayala, E., and P. G. Canonico. 1975. Aminoisobutyric acid transport in primary cultures of normal adult rat hepatocytes. *Proc. Soc. Exp. Biol. Med.* 149:1019-1022.

LITERATURE CITED

1. Glynn, A. A. 1972. Bacterial factors inhibiting host defence mechanisms. p. 75-112. *In Microbial Pathogenicity In Man and Animals* (ed. H. Smith and J. C. Pearce). Cambridge University Press, Cambridge.
2. Canonico, P. G., J. D. White, and M. C. Powanda. 1975. Peroxisome depletion in rat liver during pneumococcal sepsis. *Lab. Invest.* 33:147-150.
3. Ayala, E., and P. G. Canonico. 1975. Aminoisobutyric acid transport in primary cultures of normal adult rat hepatocytes. *Proc. Soc. Exp. Biol. Med.* 149:1019-1022.
4. de Duve, C. 1973. Biochemical studies on the occurrence, biogenesis and life history of mammalian peroxisomes. *J. Histochem. Cytochem.* 21: 941-948.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ² DA OE6429	2. DATE OF SUMMARY ³ 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUMMARY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SECY ⁴ U	6. WORK SECURITY ⁵ U	7. REGRADING ⁶ NA	8. DESIGN INSTRN ⁷ NL	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: ⁸	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER	
a. PRIMARY	62760A	3A762760A834		01	804	
b. CONTRIBUTING						
c. <i>Information</i>	CARDS 114(e)(f)					
11. TITLE (Pecase with Security Classification Code) ⁹ (U) Physiology of membrane alterations in staphylococcal enterotoxin production						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ¹⁰ 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry						
13. START DATE 74 07	14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-house	
17. CONTRACT/GRAANT		18. RESOURCES ESTIMATE		19. PERFORMING ORGANIZATION		
a. DATES/EFFECTIVE:		b. PROFESSIONAL MAN YRS EXPIRATION: NA		c. FUNDS (in thousands)		
b. NUMBER: ¹¹		FISCAL YEAR PRECEDING 76		d. CUM. AMT. CURRENT 77		
c. TYPE:						
d. KIND OF AWARD:						
e. RESPONSIBLE DOO ORGANIZATION		NAME: ¹² USA Medical Research Institute of Infectious Diseases ADDRESS: ¹³ Fort Detrick, MD 21701		NAME: ¹⁴ Pathology Division USAMRIID ADDRESS: ¹⁵ Fort Detrick, MD 21701		
f. RESPONSIBLE INDIVIDUAL		NAME: Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Punish DOD if U.S. Academic Institution) NAME: Altenbernd, R. A. TELEPHONE: 301 663-7211 SOCIAL SECURITY ACCOUNT NUMBER:		
g. GENERAL USE		Foreign intelligence considered		ASSOCIATE INVESTIGATORS NAME: Metzger, J. F. NAME: POC:DA		
21. KEYWORD (Pecase with Security Classification Code) (U) Physiology; (U) Enterotoxin, Staphylococcus; (U) Bacterial genetics;(U) Toxoids; (U) Military medicine ; (U) BW defense						
22. TECHNICAL OBJECTIVE, ¹⁶ APPROACH, PROGRESS (Punish individual paragraphs identified by number. Pecase text of each with Security Classification Code.)						
23 (U) Investigate the influence of specific biochemical alterations in composition of the bacterial cell membrane on control of synthesis and/or secretion of staphylococcal toxins, especially enterotoxin of all serotypes, since they are serious problems to the military. Study the basic mechanisms of production of the many toxic extracellular proteins produced by Staphylococcus will lead to prevention of their secretion in food products or, perhaps, in human infections and also may lead to stimulation of their production in laboratory cultures thus simplifying isolation of toxins and evaluation of their effects on experimental animals, and the development of safe, effective toxoids.						
24 (U) Study of the amounts of enterotoxin produced under a variety of experimental conditions by certain specific mutants induced in the parent strains.						
25 (U) 75 07 - 76 06 - Several hemin-deficient mutants of S. aureus were evaluated for growth and staphylococcal enterotoxin B (SEB) production. There appears to be no correlation between SEB formation and the degree of hemin supplementation. Exogenous fatty acids modify SEB production by both S. aureus 14458 and a membrane mutant derived from strain 14458. The membrane mutant is more sensitive to SEB suppression than is the parent strain 14458. Unsaturated fatty acids markedly inhibit SEB formation but do not suppress growth. Saturated fatty acids are much less inhibitory for growth and SEB production. These results are relevant to recent literature reports promoting fatty acids as food preservatives.						
Publications: Appl. Microbiol. 30:271-275, 1975. Can. J. Microbiol. 22:182-188, 1976.						

Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

* U.S. GPO: 1974-840-843/8691

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 804: Physiology of Membrane Alterations in
Staphylococcal Enterotoxin Production

Background:

Literature reports have shown that shaking rate and/or dissolved oxygen concentration are critical parameters for maximizing SEB production. Such data suggest that hemin or heme enzymes may control SEB formation and release. Membrane mutations alter SEB production several fold. The fatty acids of the cytoplasmic membrane may be involved in this mutation.

Progress:

A number of hemin-deficient mutants isolated from a strain of Staphylococcus aureus exhibited variable growth responses and SEB production when supplemented with graded amounts of hemin. There was appreciable SEB formation in the absence of exogenous hemin and it was concluded that hemin or heme enzymes are not obligately linked to SEB production.

The influence of saturated and unsaturated fatty acids on growth and SEB formation by a stock strain and a membrane mutant derived from it has been determined. The membrane mutant is much more sensitive to fatty acids than the parent but the results are otherwise similar. Lauric, myristic and palmitic acids suppress both growth and SEB formation at pH 6.0 but suppress SEB production and stimulate growth at pH 8.0. Many unsaturated fatty acids markedly suppress SEB formation while either stimulating growth or showing no effect. Results indicate that modification of the fatty acid composition of the cell cytoplasmic membrane profoundly alters SEB production while leaving growth unaffected. These observations are relevant to recent literature suggestions for the use of fatty acids as additives for food preservation.

Publications:

1. Altenbernd, R. A. 1975. Derivation of high enterotoxin B-producing mutants of Staphylococcus aureus from the parent strains. Appl. Microbiol. 30:271-275.

2. Altenbernd, R. A. 1976. Enterotoxin B formation by fermentation mutants of Staphylococcus aureus. Can. J. Microbiol. 22:182-188.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ² DA OF6411	2. DATE OF SUMMARY ³ 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SURVEY 76 02 23	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SECY ⁴ U	6. WORK SECURITY ⁵ U	7. REGRADING ⁶ NA	8. DISSEM INSTRN ⁷ NL	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: ⁸	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER	
a. PRIMARY	61101A	3A161101A91C		00	132	
b. CONTRIBUTING					834/01/805	
c. <i>for example</i>	CARDS 114(e)(f)					
11. TITLE (Procede with Security Classification Code) (U) Catecholamines and serotonin response studies to bacterial toxins						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁹ 003500 Clinical medicine; 004900 Defense; 012600 Pharmacology; 002300 Biochemistry						
13. START DATE 74 07	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house			
17. CONTRACT/GRAANT		18. RESOURCES ESTIMATE				
a. DATES/EFFECTIVE:		b. PROFESSIONAL MAN YRS PRECEDING		c. FUNDS (in thousands)		
b. NUMBER: ¹⁰ NA		FISCAL YEAR		76 1.0 20		
c. TYPE:		CURRENT		77 1.0 40		
d. KIND OF AWARD:		e. AMOUNT: F. CUM. AMT.				
19. RESPONSIBLE DOB ORGANIZATION		20. PERFORMING ORGANIZATION				
NAME: ¹¹ USA Medical Research Institute of Infectious Diseases ADDRESS: ¹² Fort Detrick, MD 21701		NAME: ¹³ Physical Sciences Division USAMRIID ADDRESS: ¹⁴ Fort Detrick, MD 21701				
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Initials SGN // U.S. Academic Institutions) NAME: ¹⁵ Bailey, P. T. TELEPHONE: 301 663-7181 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME:		POC:DA		
21. GENERAL USE Foreign intelligence considered						
22. Work Unit Summary (Procede with Security Classification Code) (U) Leukocytic endogenous mediators (LEM); (U) Central nervous system; BW defense; (U) Catecholamines; (U) Serotonin; (U) Acetylcholine; (U) Prostaglandins						
23 (U) Study effects of bacterial toxins on the storage, release, re-uptake and metabolism of the neurotransmitters in the CNS. Test and evaluate various pharmacological agents which alter neurotransmitter concentrations for possible therapeutic value. Develop a CNS model where minute quantities of purified toxins could be injected into various brain areas for the early detection of BW agents.						
24 (U) Apply stereotaxic techniques to identify the role of the CNS during bacterial infections. Use various pharmacological agents and methods for the estimation of neuro transmitters mediating the bacterial toxins effect.						
25 (U) 75 07 - 76 06 - LEM obtained from glucogen-induced peritoneal exudates in rabbits were injected into the lateral cerebral ventricle of rats to determine if LEM had a primary site of action in the CNS. LEM injected intracerebroventricularly (ICV) in the dose of 20 microliters produced hyperthermia. Heating LEM abolished this response. Endotoxin and heated endotoxin administered ICV produce delayed hyperthermia. LEM, heated LEM and saline injected ICV increased total neutrophils and decreased lymphocytes. LEM also significantly decreased plasma iron and zinc, increased plasma copper and the synthesis of plasma alpha-2-acute-phase globulin and caused flux of nonmetabolizable amino acid to the liver. LEM injected into hypothalamus and medulla oblongata produced similar but lesser results to ICV injection. Pretreatment with atropine, alpha-methyl-p-tyrosine and p-chlorophenylalanine had no effect, but they significantly inhibited synthesis of plasma acute-phase globulin. Pretreatment with indomethacin, acetylsalicylic acid and acetaminophen increased latency of hyperthermia. Their administration during hyperthermia reduced the fever response but did not alter other LEM responses. These observations suggest that systemically LEM in active doses can mediate effects via the CNS.						
Publications: Res. Commun. Chem. Pathol. Pharmacol. 11:543-552, 1975, Clin. Res. 24: 251A, 1976; Proc. Soc. Exp. Biol. Med., in press, 1976.						

BODY OF REPORT

Project No. 3A061101A91C: In-House Laboratory Independent Research (U)
(3A762760A834):

Task No. 3A061101A91C 00:
(3A762760A834 01): (Pathogenesis of Infection of Military Importance)

Work Unit No. 91C 00 132: Catecholamines and Serotonin Response Studies to
(834 01 805): Bacterial Toxins

Background:

LEM obtained from a glycogen-induced peritoneal exudates in rabbits has been shown to lower plasma Fe and Zn concentrations, increase plasma Cu, enhance the flux of amino acids from serum to liver, cause the release of marrow neutrophils and increase the synthesis of acute-phase globulins following systemic administration in the rat.^{1,2} Intracisternal injections of LEM into rats caused a lowering in plasma Fe concentration and elevated neutrophil levels in very minute doses.³ Rabbits produced a marked hyperthermia to an intracisternal administered dose of LEM which was ineffective systemically.⁴ These metabolic alterations following central administration of LEM suggest a primary site of action in the central nervous system.

Progress:

In order to clarify if LEM has a primary site of action in the central nervous system (CNS) a surgical procedure was set-up to permanently implant rats with guide cannula (Plastic Products Co., VA) in the right lateral cerebral ventricle. This procedure permits intracerebroventricular (ICV) injection of various agents into the CNS of unanesthetized and unrestrained laboratory animals.

LEM injected ICV in the dose of 10, 20 or 50 μ l was observed to produce rapid hyperthermia (2-3 C) within 60 min of each injection; these doses were ineffective when administered IP or IV. The hyperthermia was long-lasting but did not exceed 24 hr. Heated LEM (supernatant, boiled for 30 min; Δ LEM) and saline did not alter normal rectal temperature. Endotoxin and heated endotoxin (supernatant, boiled for 30 min; Δ endotoxin) both exhibited a delayed hyperthermia when compared to LEM. Since heat-treatment abolished the hyperthermia produced by LEM but not that of endotoxin, it was suggested that the LEM preparation was not contaminated with endotoxin.

LEM, Δ LEM, and saline injected ICV were observed to cause a nonspecific increase in neutrophils and a decrease in lymphocyte concentrations.

Alterations in serum Zn, Fe and Cu concentrations after systemic injections of LEM have been well documented in rats. The present study for the first time indicates that an ICV injection of LEM causes a decrease in serum Zn and Fe and an increase Cu concentration. ICV-administered LEM leads to a heretofore undescribed CNS role in the elevation of an acute-phase protein α_2 -macrofetoprotein (α_2 -MFP) concentration and a marked hepatic intracellular accumulation of a nonmetabolizable amino acid, [14 C]AIB. Thus, indicating that LEM can act via a CNS mechanism in stimulating the hepatic transport of amino acids and the synthesis of plasma α_2 -MFP. A manuscript has been written and submitted for publication.

Many investigators have shown that the monoamines (norepinephrine and serotonin) and acetylcholine present in the CNS are concerned with thermoregulation. In order to examine the role of the catecholamines following ICV injections of LEM, α -methyl-p-tyrosine (tyrosine hydroxylase inhibitor which significantly decreases catecholamine levels: α -MPT) was administered. α -MPT caused a significant inhibition of the release of α_2 -MFP and did not alter the neutrophil, lymphocyte, Zn and temperature responses to ICV-injected LEM. p-Chlorophenylalanine (an agent which significantly lowers tissue serotonin concentrations; PCPA) and atropine (an anticholinergic agent) produced effects similar to α -MPT. The observed decrease in plasma concentrations of α_2 -MFP by α -MPT, PCPA, and atropine after LEM suggest that a balance between the putative neurotransmitters is needed for this response and that it is unlikely that the hyperthermic effect of LEM is mediated by alteration in the levels of monoamines and acetylcholine in various brain areas.

Recent evidence has implicated prostaglandins (PG) as the mediator during the hyperthermic response to leukocytic mediators in various laboratory animals. The hyperthermic response to LEM in the present study did not appear to be mediated via an increase in PG synthesis since indomethacin, acetaminophen, and aspirin (inhibitors of PG synthesis) did not completely abolish the hyperthermia. Although, pretreatment with these inhibitors did delay the hyperthermia and if injected during the hyperthermia a slight reduction was observed, suggesting that PG may be involved.

The findings that LEM produced hyperthermia when administered into the cerebral ventricles was further examined by injections into the preoptic/anterior hypothalamic area (PO/AH), medulla oblongata, and the anterior hypothalamus region. These brain areas have also been shown to control body temperature. The present findings indicated that LEM (2 μ l) injected into other brain regions which are known to be thermoresponsive can produce slight hyperthermia. Since Zn and α_2 -MFP were not altered by ICV injections of LEM in these particular structures, other CNS areas may be involved for these responses.

Publications:

1. Bailey, P. T., and D. T. George. 1976. Mechanisms of L-dopa induced alterations in glucagon/carbohydrate metabolism. *Clin. Res.* 24:251A.
2. Bailey, P. T., F. B. Abeles, E. C. Hauer, and C. A. Mapes. 1976. Intracerebroventricular administration of leukocytic endogenous mediators (LEM) in the rat. *Proc. Soc. Exp. Biol. Med.* In press.

LITERATURE CITED

1. Pekarek, R., R. Wannemacher, M. Powanda, F. Abeles, D. Mosher, R. Dinterman, and W. Beisel. 1974. Further evidence that leukocytic endogenous mediator (LEM) is not endotoxin. *Life Sci.* 14:1765-1776.
2. Beisel, W. R., and R. S. Pekarek. 1972. Acute stress and trace element metabolism. *Int. Rev. Neurobiol. Suppl.* 1:58-62.
3. Kampschmidt, R. F., H. F. Upchurch, and L. A. Pulliam. 1973. Investigations on the mode of action of endogenous mediator. *Proc. Soc. Exp. Biol. Med.* 143:279-283.
4. Adler, R. D., and R. J. T. Joy. 1965. Febrile responses to the intracisternal injection of endogenous (leukocytic) pyrogen in the rabbit. *Proc. Soc. Exp. Biol. Med.* 119:660-663.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL
3. DATE PREV SURVEY 75 07 31	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCRTY ^c U	6. WORK SECURITY ^c U	DA OF6422	76 07 01	DD-DR&E(AR)636
10. NO./CODES: ^d a. PRIMARY 62760A	PROGRAM ELEMENT PROJECT NUMBER 3A762760A834			7. REGRADING ^d NA	8. DRG'DN INSTR'N NL	9. SPECIFIC DATA-CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
b. CONTRIBUTING				10. TASK AREA NUMBER 01	11. LEVEL OF SUM A. WORK UNIT WORK UNIT NUMBER 806	
c. <i>Information</i> CARDS 114(e)(f)						
12. TITLE (Provide with Security Classification Code) (U) Role of bacterial exotoxins in disease pathogenesis						
13. SCIENTIFIC AND TECHNOLOGICAL AREAS ^e 003500 Clinical medicine; 004900 Defense; 002600 Biology (Pathology)						
14. START DATE 75 07	15. ESTIMATED COMPLETION DATE CONT	16. FUNDING AGENCY DA		17. PERFORMANCE METHOD C. In-house		
18. CONTRACT/GRAANT a. DATES/EFFECTIVE: b. NUMBER: c. TYPE: d. KIND OF AWARD:		19. RESOURCES ESTIMATE EXPIRATION: FISCAL YEAR 76 CURRENT 77		20. PROFESSIONAL MAN YRS 1.0 1.0		21. FUNDS (in thousands) 137 143
22. RESPONSIBLE DOD ORGANIZATION NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		23. PERFORMING ORGANIZATION NAME: Pathology Division USAMRIID ADDRESS: Fort Detrick, MD 21701		24. PRINCIPAL INVESTIGATOR (Provide name if U.S. Academic institution) NAME: Leppla, S. H. TELEPHONE: 301 663-7211 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: Muehl, L. A. NAME: POC:DA		
25. GENERAL USE Foreign intelligence considered						
26. REVIEWS (Provide each with Security Classification Code) (U) Exotoxins; (U) Pseudomonas aeruginosa; (U) Burn infections; (U) Military medicine; (U) Laboratory animals; (U) Diphtheria toxin; (U) BW defense						
27. TECHNICAL OBJECTIVE, 28. APPROACH, 29. PROGRESS (Provide text of each with Security Classification Code.)						
23 (U) Characterize certain potent bacterial exotoxins and identify their role in infection. The exotoxin used as a model, <i>Pseudomonas aeruginosa</i> , is believed to be responsible for the high frequency and severity of <i>Pseudomonas</i> infections of burn wounds. Both the bacterium and its exotoxin are potential BW agents. Characterization of this exotoxin will improve our ability to prevent, diagnose, and treat bacterial infections or intoxications, and deal with novel uses of these and other pathogenic agents.						
24 (U) After characterizing and developing an assay for exotoxins in mice, examine the biological factors involved in toxin synthesis and pathogenesis.						
25 (U) 75 07 - 76 06 - The exotoxin (PE) of <i>P. aeruginosa</i> was purified from 50-L cultures, yielding 100 mg of homogeneous protein. The protein has a molecular weight of 66,000 daltons, an isoelectric point of 5.1, N-terminal arginine, no free sulphydryls, and 4 disulfide bridges. The median lethal dose was 0.1 microgram in mice and 20 micrograms in rats. PE is a proenzyme; simultaneous reduction and denaturation causes a 20- to 50-fold increase in ADP-ribosylation activity without alteration of the molecular weight. Treatment of denatured PE with proteases produces peptide fragments retaining ADP-ribosylation activity. Evidence that PE may contribute to virulence included demonstrations that (1) nearly all <i>P. aeruginosa</i> strains are toxinogenic and (2) animals are frequently exposed to PE in subclinical infections. A toxoid was prepared which induced immunity to PE in mice.						
Publication: Infect. Immunity 14: in press, 1976.						
Available to contractors upon originator's approval.						

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Aspects of Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 806: Role of Bacterial Exotoxins in Disease Pathogenesis

Background:

Infection by Pseudomonas aeruginosa is a frequent and serious complication in debilitated patients, particularly in those whose phagocytic functions are impaired. An exotoxin which may account for the special virulence of P. aeruginosa in these patients was recently described.^{1,2} This exotoxin (PE) was shown to be lethal to a variety of experimental animals and cultured cell lines. One laboratory has reported that passive immunization against PE protects mice from lethal challenge by P. aeruginosa,² suggesting that PE is an important virulence factor and offering hope that immunization against PE might aid in control of human infections.

A substantial advance in understanding the mechanism of action of PE, reported during the last year, was the demonstration³ that PE, like diphtheria toxin (DE), acts by blocking protein synthesis. Both these toxins catalyze transfer of the adenosine diphosphate (ADP) ribose portion of nicotinamide adenine dinucleotide (NAD) to mammalian elongation factor 2 (EF-2). The "ADP-ribosylation"⁴ of EF-2 renders it unable to cause translocation of ribosomes along mRNA during protein synthesis.

Progress:

Since PE was only recently described and had not been previously studied in this Institute, it was necessary to develop methods for its preparation and assay. Initially several small samples of PE were purified by combinations of published procedures, with detection of PE being achieved in mouse lethality tests. Injection of this material into a pony produced a high titered anti-toxin, which is now used routinely in a radial diffusion test (Mancini technique) to measure PE, and in Elek tests to identify Pseudomonas strains which produce PE. Lower concentrations of PE (1-10 ng/ml) can be detected in the cell culture cytotoxicity test developed by Dr. John Middlebrook (Work Unit 834 01 807). More recently an assay of ADP-ribosylation capable of measuring 0.2-2 µg/ml of PE has been established.

Many of the studies we planned required relatively large amounts of PE. After testing several purification procedures we adopted a simple protocol involving batch adsorption to DEAE-cellulose and gradient chromatography on DEAE-cellulose and hydroxylapatite. Several 50-L cultures processed in this way yielded > 100 mg of pure PE. Table I summarizes a typical preparation.

TABLE I. PURIFICATION OF PSEUDOMONAS AERUGINOSA EXOTOXIN

PURIFICATION STAGE	TOTAL		TOTAL TOXIN		SPECIFIC ACTIVITY	TOTAL CARBOHYDRATES (mg)
	VOLUME (ml)	PROTEIN (mg)	BY RADIAL DIFFUSION (mg)	BY RIBOSYLA-TION (mg)		
Culture supernatant	50,000	5,500	≤ 500	350	6	28,000
DEAE eluate	2,000	500	190	180	35	60
DEAE peak	150	166	150	140	85	2
Hydroxylapatite peak	30	135	135	135	100	≤ 0.15

The purity of the PE preparations was tested by electrophoresis in polyacrylamide gels. Disc and SDS gels yielded single bands while isoelectric focusing gave, in addition, a few minor bands comprising < 5% of the total. In Ouchterlony gel diffusion the purified PE gave a single precipitin line, which did not interact with a diphtheria toxin-antitoxin reaction. Analysis with anthrone reagent and gas chromatography indicated the absence of carbohydrate. Amino acid analysis revealed a relatively normal composition which has no apparent similarity to that of DE. A dansyl chloride method showed that the N-terminal amino acid was arginine. Use of ^{14}C -iodoacetamide demonstrated that PE contains no free sulphydryls and 4 disulfides. The latter value is slightly uncertain due to the low degree of precision in the $E_{280}^{1\%}$ and lack of agreement between 2 protein standards. The MW determined by electrophoresis in SDS-polyacrylamide gels is 66,000. This is significantly higher than the values (50,000-54,000) obtained in other laboratories using gel filtration. The isoelectric point (pI) was found to be 5.1. The LD₅₀ of the preparation was 0.1 µg in mice weighing 20 gm and 20 µg in rats weighing 350 gm.

Diphtheria toxin, which acts within sensitive cells by the same mechanism as PE, is probably the best characterized bacterial toxin. The detailed understanding of its structure-activity relationship⁴ has allowed the design of preventive and therapeutic measures to be based on a logical framework. With the expectation that similar benefits would be obtained in the case of PE, detailed study was made of the relation of structure to ADP-ribosylation activity. Like Iglesias and Kabat,³ we found that

apparently monodisperse PE samples possess ADP-ribosylation activity. Most preparations of DE also have enzymatic activity, but the amount correlates exactly with the amount of a 24,000-dalton peptide fragment denoted "A." It has been shown that the intact, 63,000-dalton DE is a proenzyme having no enzymatic activity. The active species, fragment A, can be generated from the 63,000-dalton protein by cleavage of a peptide bond and reduction of a disulfide bond. When PE, treated with reducing agents, was electrophoresed on SDS-polyacrylamide gels essentially all the ADP-ribosylation activity coincided with the 66,000 dalton species. This proved that in PE the activity of the untreated preparation was a property of the intact protein and not of a peptide fragment.

Another step toward understanding the structure of PE came with the demonstration that PE, like DE, is a proenzyme. It was found that the ADP-ribosylation activity of PE could be increased 20- to 50-fold by simultaneous treatment with reducing and denaturing agents. This treatment increased the activity of PE approximately to the level of an equimolar amount of DE fragment A. SDS gels containing internal fluorescent protein markers showed that activation of PE did not involve a decrease in MW; the ADP-ribosylation activity still coincided with the 66,000-dalton species. It has been concluded that activation involves cleavage of disulfide bonds and a conformational change which exposes the previously buried active site. This model predicts that it should be possible to reverse activation by careful reoxidation of sulfhydryl groups. Attempts to achieve reversal using a variety of incubation conditions have failed.

To correlate activation with the degree of disulfide bond reduction, PE was incubated in dithiothreitol with varying concentrations of urea or guanidine-HCl and aliquots removed for assay of ribosylation activity and newly formed sulfhydryl groups. Either 1 M guanidine-HCl or 4 M urea caused complete activation as well as reduction of all 4 disulfides, indicating that the rearrangement in structure which constitutes activation is a concerted event.

Though activation does not require peptide bond scission, some evidence from this and other laboratories indicated the existence of enzymatically active peptide fragments analogous to fragment A of DE. Recent work has shown that when PE is activated or denatured it becomes especially sensitive to proteases. Incubation with trypsin, an extracellular protease of P. aeruginosa, or a mouse liver lysosomal extract generated active fragments of several sizes demonstrable on SDS gels. Since these active fragments have been generated only from activated PE, which already possesses full enzymatic activity, no biological significance can be attached to their formation. Analyses of PE recovered from intoxicated cells will be necessary in order to determine whether activation proceeds through the conformation change or fragmentation mechanisms.

Several studies were directed toward determining whether PE is a major virulence factor. A group of 80 clinical isolates of P. aeruginosa was screened for in vitro production of PE using the Elek and the cell culture cytotoxicity tests. In excess of 90% of the isolates were found to make toxin, a result consistent with the hypothesis that Pseudomonas toxin is an important virulence factor. In another study a sensitive HA assay was used to measure antibodies to PE. A number of normal sera from animals and humans were found to have low titers of antibody. It is probable that these antibodies were induced during subclinical infections. The presence of these antibodies indicates that PE is made in vivo, and thereby supports the view that PE is a virulence factor.

The most definitive proof that PE contributes to virulence would be demonstration that immunity to PE protects against challenge with live P. aeruginosa. Preliminary tests have been made in animals immunized either passively or actively. In an attempted repetition of the published passive protection experiment,² the high titer pony antitoxin, a normal horse serum, and saline were injected into mice IP followed 2 hr later by live Pseudomonas. Both sera protected when compared to saline. This suggested that protection was due to "natural" antibodies or to stimulation of the RE system. A more extensive passive immunization experiment was therefore performed in which portions of normal horse and immune pony sera were pretreated by heat-inactivation or adsorption to the challenge bacteria. The pony antitoxin, whether adsorbed or not, appeared to confer a small degree of protection while the normal horse immunoglobulin did not.

To induce active immunity a toxoid was prepared by reaction of purified PE with glutaraldehyde. The toxoid, administered to mice in two doses, 100- μ g, induced resistance to subsequent challenge with at least 10 LD₅₀ of PE (Table II).

TABLE II. PROTECTIVE IMMUNITY INDUCED BY GLUTARALDEHYDE TOXOID

GROUP	TOXOID DOSE (μ g)	% SURVIVAL FOLLOWING CHALLENGE DOSE (LD ₅₀)			
		0.3	1	3	10
1	None, PBS	100	0	0	0
2	2 x 10	100	100	75	75
3	2 x 100	100	100	100	100

These mice were later rechallenged with 50 LD₅₀ of PE; most survived. Immunization alone did not induce antibodies to PE detectable in the HA assay, but immunized mice surviving challenge with PE showed titers as high as 1:5120. This suggests that glutaraldehyde may have destroyed important antigenic sites and that other methods of toxoiding might yield more effective immunogens. Preliminary tests in small numbers of mice failed to demonstrate that active immunization protects mice from IP challenge by P. aeruginosa.

Several lines of argument suggest that the contribution of PE to virulence may be greater in burn wound infections than in systemic infections. Tests of several types of burn wounds in mice led to tentative selection of a brass block burn method, which causes no mortality unless followed by seeding with P. aeruginosa. After determination of an appropriate challenge dose, the resistance of immune and naive mice to infection through the burn wound will be compared.

Publication:

Leppla, S. H. 1976. Purification and characterization of the exotoxin of Pseudomonas aeruginosa. Infect. Immun. 14: in press.

LITERATURE CITED

1. Liu, P. V., S. Yoshii, and H. Hsieh. 1973. Exotoxins of Pseudomonas aeruginosa. II. Concentration, purification, and characterization of exotoxin A. J. Infect. Dis. 128:514-519.
2. Liu, P. V., and H. Hsieh. 1973. Exotoxins of Pseudomonas aeruginosa. III. Characteristics of antitoxin A. J. Infect. Dis. 128:520-526.
3. Iglewski, B. H., and D. Kabat. 1975. NAD-dependent inhibition of protein synthesis by Pseudomonas aeruginosa toxin. Proc. Nat. Acad. Sci. 72:2284-2288.
4. Collier, R. J. 1975. Diphtheria toxin: mode of action and structure. Bacteriol. Rev. 39:54-85.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ³	2. DATE OF SUMMARY ³	REPORT CONTROL SYMBOL	
3. DATE PREV SUMRY 75 12 31	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY ³ U	6. WORK SECURITY ³ U	7. REGRADING ³ NA	8. DOD/INSTRN ³ NL	9. SPECIFIC DATA-CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT
10. NO./CODES: a. PRIMARY b. CONTRIBUTING c. Foreign funding		PROGRAM ELEMENT 62760A		PROJECT NUMBER 3A762760A834	TASK AREA NUMBER 01	WORK UNIT NUMBER 807	
11. TITLE (Pencode with Security Classification Code) (U) Mechanism of action of bacterial exotoxins							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ³ 003500 Clinical medicine; 004900 Defense; 016800 Toxicology; 002300 Biochemistry							
13. START DATE 75 11	14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house			
17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS		20. FUNDS (in thousands)	
a. DATES/EFFECTIVE: b. NUMBER: c. TYPE: d. KIND OF AWARD:		EXPIRATION: NA	FISCAL YEAR PRECEDING CURRENT	76 0.5	68.5		
		4. AMOUNT: e. CUM. AMT.		77 1.0	156.0		
21. RESPONSIBLE DOD ORGANIZATION		22. PERFORMING ORGANIZATION					
NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		NAME: Pathology Division USAMRIID ADDRESS: Fort Detrick, MD 21701					
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Punish each with Security Classification Code) NAME: Middlebrook, J. L. TELEPHONE: 301 663-7211 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME:					
23. GENERAL USE Foreign intelligence considered		POC:DA					
24. KEY WORDS (Pencode EACH with Security Classification Code) (U) Exotoxins; (U) Food poisoning; (U) Infected burns; (U) Staphylococcus; (U) Pseudomonas; (U) Military medicine; (U) BW defense							
25. TECHNICAL OBJECTIVE, 26. APPROACH, 27. PROGRESS (Punish individual paragraphs identified by number. Pencode text of each with Security Classification Code.)							
23 (U) Elucidate the mechanisms of action of militarily significant bacterial exotoxins in order to develop prophylactic or therapeutic measures. Two toxins are under investigation: <i>Pseudomonas aeruginosa</i> exotoxin A (PE), which has been implicated as a (the?) causative agent in the mortality of recuperating burn casualties who develop <i>Pseudomonas</i> infections; staphylococcal enterotoxin B (SEB) which has a demonstrated BW potential.							
24 (U) After finding a susceptible cell or organ tissue for each toxin, attempt to block by drug or chemical treatment the action of a toxin. Test positive findings in laboratory animals.							
25 (U) 75 07 - 76 06 - Using cultured mammalian cells the mechanisms of action of <i>Pseudomonas aeruginosa</i> exotoxin (PE) and staphylococcal enterotoxin B (SEB) were studied. <i>Pseudomonas</i> exotoxin was cytotoxic for 22 cell lines examined. The response of cells was quantitated by the development of a cytotoxic assay system. The potencies of PE and diphtheria exotoxin (DE) were found to be much different for the cell lines examined. Many drugs were tested and, although some protected cells from DE, none had any protective effects on PE's cytotoxicity. We conclude that the mechanisms of action of DE and PE on intact cells are different.							
The action of SEB on cultured cells was also studied and no response could be observed despite claims to that effect in the literature. However, NAD, an important cofactor for many other bacterial exotoxins, was found to bind to SEB and the bio-physical chemistry of this binding is under investigation.							
Publication: Fed. Proc. 35:1394, 1976. Infect. Immun. 14: in press (2), 1976.							

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 807: Mechanism of Action of Bacterial Exotoxins

Background:

Many of the harmful effects of bacterial infections are believed to be mediated by exotoxins elaborated by the organism. An understanding of the mechanism(s) of action of these toxins might lead to insights suggesting a particular course of treatment. Thus, it is our purpose to study the mechanism(s) of action of militarily significant bacterial exotoxins and also to test available drugs for their therapeutic potential. Two toxins are currently under investigation: staphylococcal enterotoxin B (SEB) and Pseudomonas aeruginosa exotoxin A.

SEB is one of a number of enterotoxins elaborated by Staphylococcus aureus. These toxins are believed to be responsible for most of the symptoms of food poisoning where S. aureus is the causative agent. Although much is known about the chemistry and properties of this toxin,¹ the mechanism by which it induces emesis and diarrhea is unclear.

The second exotoxin of interest is one elaborated by P. aeruginosa. This normally harmless bacterium represents a severe threat to patients with impaired phagocytic activity. Indeed, Pseudomonas sepsis contributes significantly to mortality figures for severely burned patients² as well as those with hematologic neoplasms.³ Although Pseudomonas produces a variety of extracellular substances, there is evidence to suggest that an exotoxin originally described by Liu⁴ might be the agent responsible for the serious symptoms of infection. Little was known about Pseudomonas exotoxin (PE) until it was recently demonstrated that it and diphtheria exotoxin (DE) have similar in vitro enzymatic activities.⁵ This finding opened up the possibility that the 2 toxins also have similar mechanisms of action on intact cells.

Progress:

Part I - PE. We began with an examination of the effects of PE on a number of different mammalian cell lines in culture. Our initial observation was that the addition of PE caused cells to round up and eventually lyse. Measurement of this cytotoxic response was qualitative at best so an assay system was developed whereby the response could be made more quantitative. This assay system has evolved to where it is simple, reliable, quantitative, readily adaptable to most cell lines, and could be used to study the cytotoxic

effects of nearly any toxin. Briefly, the assay consists of seeding multiwell tissue culture plates with the cell line of interest, incubating the cells for 24 hr, then adding the toxin. After 48 hr the wells are washed with a balanced salt solution; those cells remaining are dissolved in NaOH for a Lowry protein determination. Although this methodology lends itself well to the measurement of macromolecular synthesis or other cellular metabolic functions, we find that a simple protein analysis correlates well with other measures of cell viability. Since the Lowry protein assay has been automated at this Institute, we have adopted this particular measurement for our cytotoxic assay.

Using the above methodology we investigated many variables of the system (times of toxin-cell incubation, seeding by trypsinization or scraping, different media, different sera, seeding density, etc.) that we believed might have an effect on work with PE and found that only one, serum type, resulted in artifactual results. Calf, horse, and human serum all protected the cells to various degrees from the cytotoxic effects of PE. Fetal calf serum seemed to be neutral for purposes of our assay and was used for all subsequent work. By the use of serum mixing experiments, HA assay, and the $(\text{NH}_4)_2\text{SO}_4$ precipitation of antibody (Farr) test we determined that the protective effect of the sera is most likely due to low levels of PE-specific antibody. This determination along with the protocol for our cytotoxicity assay was submitted for publication in Infection and Immunity.

During the course of the work described above, Iglewski and Kabat⁵ published data which indicated that PE and DE have similar enzymatic activities when examined in cell-free preparations. This finding raised the distinct possibility that PE has the same mechanism of action in cells or animals as does DE. We now have several lines of evidence suggesting that such is not the case.

In some initial tests, we found that PE-specific antibody would not protect cells from the cytotoxic effect of DE nor would DE-specific antibody protect cells from PE (Table I).

TABLE I. PROTECTION OF CELLS BY PE- AND DE- SPECIFIC ANTISERA

GROUP	CELL PROTEIN (mg/100 ml)	
	PE	DE
Control	26.0	27.7
Toxin (0.1 µg/ml)	0.7	0.5
Toxin + anti-PE	23.6	0.5
Toxin + anti-DE	0.3	28.7

This result indicates that the 2 toxins are not identical. Moreover, in an experiment where the antigen-antisera ratios were varied over a wide range we could detect no cross-reactivity at all. This observation makes it highly unlikely that the 2 toxins have any similar antigenic determinants, i.e. exposed conformations are different. Our notion that PE and DE have different conformations received support from some other studies where we found that the circular dichroic spectra of PE and DE are very different.

In another large series of experiments we have determined the dose-response of many cell lines to both PE and DE (Table II).

TABLE II. SENSITIVITY OF CELLS TO PE AND DE

SPECIES SOURCE OF CELL	CELL TYPE OR LINE	TISSUE CULTURE LD_{50} (ng/ml)	
		PE	DE
Human	CC1-6	0.7	0.2
	WI-38	1.5	0.2
	HeLa	15.0	1.5
	KB	3.0	0.3
	WISH	9.0	0.7
	HeP-2	8.0	0.3
Monkey	MK-2	3.0	0.02
	Vero	15.0	0.01
	BS-C-1	50.0	0.01
Hamster	HAK	10.0	0.08
	BHK-21	5.0	0.2
Chicken	Primary chick fibroblasts	2.0	0.15
Rat	CC1-107	3.0	300.00
Mouse	L929	0.1	> 1000.00
	3T3	0.15	> 1000.00
	Neuroblastoma	10.0	1000.00

We cannot detect any similarity in cell line sensitivity to the 2 toxins on either a relative or an absolute scale. We believe these data present strong evidence that the 2 toxins do not operate in an entirely analogous manner and have submitted the above findings for publication in *Infection and Immunity*.

After defining the potency of PE and DE with cultured cells, we moved on to test the ability of various chemicals or drugs to protect cells from either toxin. We adopted this approach for 2 reasons: First, if a drug can be found which protects cells from the toxins, it or a congener might have direct therapeutic potential. Secondly, since many of the drugs or chemicals investigated were known to block particular cellular processes or functions, an effect on the toxin-induced cytotoxicity might provide evidence that the process blocked by the drug is somehow involved in the toxin's mechanism of action. Table III lists the drugs investigated, the cell line(s) used, and the effect on toxin-induced cytotoxicity.

TABLE III. EFFECT OF SELECTED DRUGS AND CELL LINES ON TOXIN-INDUCED CYTOTOXICITY

AGENT	CELL LINE(S)	PROTECTION ^a	
		PE	DE
NH_4Cl	L929	0	ND
	HeLa	0	++
	HeP-2	0	++
NaF	L929	-	ND
	HeLa	-	++
	HeP-2	-	++
KCN	L929	0	ND
	HeLa	0	+
	HeP-2	0	+
Dinitrophenol	HeP-2	-	+
p-Chloromercuribenzoic acid	HeP-2	0	+
Sodium arsenite	HeP-2	-	++
Colcemid	HeP-2	0	+
Colchicine	HeP-2	0	0
Ouabain	HeP-2	0	0
Ruthenium red	HeP-2	0	+
Cytochalasin B	HeP-2	0	+
Poly-L-ornithine	HeLa	0	+
	HeP-2	0	+

^a

+ (moderate level of protection).

++ (high level of protection).

0 (no effect).

- (potentiation of cytotoxicity).

ND, not done.

NH_4Cl has been previously shown to protect HeP-2 cells from DE; in its presence, the toxin appears to bind normally to the cell membrane but internalization is somehow prevented. We did not observe any protection when NH_4Cl was present during PE challenge of cells.

Sodium fluoride, KCN, and dinitrophenol (DNP) all are believed to block cellular energy production. All 3 drugs protected cells from DE but either had no effect on (DNP, KCN) or potentiated PE's cytotoxicity (NaF).

The sulfhydryl reactive reagent p-chloromercuribenzoic acid did not alter PE's cytotoxicity but protected cells from DE. Another related agent, sodium arsenite, inhibits disulfide reductases. We found this agent very effective in protecting cells from DE while it potentiated PE's cytotoxicity.

Colcemid and colchicine, which affect the assembly of microtubules, were found to have no effect when cells were challenged with PE. Colcemid had a modest protective effect when DE was the challenge toxin. Cytochalasin B, another drug which disturbs a component of the cytoskeleton (microfilaments), exhibited a protective effect against DE's cytotoxicity but had no effect against PE's.

Poly-L-ornithine is reported to increase cellular rates of pinocytosis. We tested poly-L-ornithine and found that it would protect against DE, but not PE, challenge.

Finally, we tested inhibitors of the membrane ATPases. Ouabain, an inhibitor of the Na^+ , K^+ -dependent ATPase, had no effect on either toxin's cytotoxicity. Ruthenium red, an inhibitor of the Ca^{++} , Mg^{++} -dependent ATPase, protected cells from DE while having no effect with PE.

Some additional drugs are now being tested and when completed, the combined results will be submitted for publication. We believe our drug protection studies combined with the cell sensitivity studies present conclusive evidence that PE and DE have different mechanisms of action on intact cells.

Part II. SEB. Work with SEB has been mostly a disappointment with one bright spot. We have spent much time and effort trying to reproduce the work of Schaeffer and Gabliks.¹ However, we have been unable to do so even after some personal conversations with Schaeffer. We have recently found that a group in England has also tried (unsuccessfully) to repeat this work. Based on this information and our own work we have reached the conclusion that the published results of Schaeffer and Gabliks may not be reliable.

Because other bacterial toxins have been reported to be NAD^+ -dependent, we have attempted to determine if NAD^+ might be important for SEB as well. We have preliminary results which demonstrate that NAD does bind to SEB with at least a moderate affinity constant. We are pursuing this line of investigation in hopes that NAD^+ might be a cofactor for SEB's action.

In some related work (Work Unit 834 01 806) the circular dichroism (CD) of SEC and some of its derivatives were measured as well as the kinetics of unfolding (again using CD). The results indicated that (1) SEC has a very low fraction of α -helical structure and a moderate amount of β , (2) that "nicking" the toxin with trypsin does not lead to any substantial conformational changes, but that (3) the guanidine-HCl-induced unfoldings of the intact and "nicked" toxin are different.

Presentation:

Middlebrook, J. L., and R. B. Dorland. Response of mammalian cells to the exotoxins of Corynebacterium diphtheriae and Pseudomonas aeruginosa: differential cytotoxicity. Presented, American Society for Biological Chemists, San Francisco, CA, 7 June 1976, (Program and abstract, no. 188). (Fed. Proc. 35:1394, 1976).

Publications:

1. Middlebrook, J. L., and R. B. Dorland. 1976. Serum effects on the response of mammalian cells to exotoxins of Pseudomonas aeruginosa and Corynebacterium diphtheriae. Infect. Immun. 14: in press.
2. Middlebrook, J. L., and R. B. Dorland. 1976. Response of cultured mammalian cells to the exotoxins of Pseudomonas aeruginosa and Corynebacterium diphtheriae: differential cytotoxicity. Infect. Immun. 14: in press.

LITERATURE CITED

1. Bergdoll, M. S. 1970. Enterotoxins. pp. 265-326. In Microbial Toxins, Vol. III (T. C. Montie, S. Kadis, and S. J. Ajl, ed.). Academic Press, New York.
2. Pruitt, B. A., Jr. 1974. Infections caused by Pseudomonas species in patients with burns and in other surgical patients. J. Infect. Dis. 130:S8-S13.
3. Tapper, M. L., and D. Armstrong. 1974. Bacteremia due to Pseudomonas aeruginosa complicating neoplastic disease: a progress report. J. Infect. Dis. 130:S14-S23.
4. Liu, P. V. 1966. The roles of various fractions of Pseudomonas aeruginosa in its pathogenesis. III. Identity of the lethal toxins produced in vitro and in vivo. J. Infect. Dis. 116:481-489.
5. Iglesias, B. H., and D. Kabat. 1975. NAD-dependent inhibition of protein synthesis by Pseudomonas aeruginosa toxin. Proc. Nat. Acad. Sci. 72:2284-2288.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ²	2. DATE OF SUMMARY ³	REPORT CONTROL SYMBOL
3. DATE PREV SUMMARY 76 01 19	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCRTY ⁴ U	6. WORK SECURITY ⁴ U	DA OB6410	76 07 01	DD-DR&E(AR)636
10. NO./CODES ⁵	PROGRAM ELEMENT 62760A	PROJECT NUMBER 3A762760A834	TASK AREA NUMBER 02	7. REGRADING ⁶		
8. PRIMARY				8A. DISPN INSTRN ⁷ NA	8B. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	8C. LEVEL OF SUM A. WORK UNIT
9. CONTRIBUTING				9. WORK UNIT NUMBER 002		
c. EQUIPMENT CARDS 114(e)(f)						
11. TITLE (Provide with Security Classification Code) (U) Evaluation of experimental vaccines in man in BW defense research						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁸ 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry						
13. START DATE 61 10	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house			
17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS 0	20. FUNDS (in thousands) 0	
a. DATES/EFFECTIVE:	EXPIRATION:	FISCAL YEAR	CURRENT	76	0	
b. NUMBER: NA	c. TYPE:	d. AMOUNT:		77	0	
e. KIND OF AWARD:	f. CUM. AMT.					
19. RESPONSIBLE DOO ORGANIZATION		20. PERFORMING ORGANIZATION				
NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		NAME: Medical Division USAMRIID ADDRESS: Fort Detrick, MD 21701				
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Junior Dean // U.S. Academic Institution) NAME: Ascher, M. S. TELEPHONE: 301 663-7361 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: Harber, P. H. NAME: POC:DA				
21. GENERAL USE Foreign intelligence considered						
22. KEYWORDS (Provide each with Security Classification Code) (U) BW defense; (U) Military medicine; (U) Vaccines; (U) Immunization; (U) Human volunteers; (U) Encephalitis, equine (VEE,WEE,EEE);Q fever						
23. TECHNICAL OBJECTIVE, ⁹ 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Provide text of each with Security Classification Code.)						
23 (U) Evaluate experimental vaccines developed by various contractors, organizations or other governmental agencies. This work unit is an essential element in a comprehensive program for medical defense against BW agents because it covers the initial testing in man of newly developed experimental vaccines.						
24 (U) Test vaccines are given to experimental animals, and when considered safe, to volunteers. Vaccinees are followed to evaluate the clinical and laboratory responses to vaccination and to assess the vaccine's protective efficacy in preventing laboratory-acquired infections.						
25 (U) 75 07 - 76 06 - Administration of vaccines to at-risk laboratory personnel has continued. Serum neutralizing antibody to VEE virus subtypes IA, IB, and IC was shown to persist for 7 to 9 yr in 19 of 20 volunteers immunized with TC-83 vaccine (subtype IA). Fewer of these 20 volunteers had antibody detectable against more antigenically remote subtypes ID, IE, II, III, and IV. The antigenically restricted nature of the serological response after TC-83 vaccine was confirmed in a group of 10 laboratory workers who were bled and titered against VEE subtypes 28 days after vaccination. Booster vaccination of persons previously immunized against VEE, WEE and EEE viruses suggests that EEE and WEE vaccines have only a minimal effect, if any, on preexisting VEE neutralizing antibody titers. Febrile vaccine reaction after TC-83 vaccine is associated with the emergence and recovery of VEE virus in the nasopharynx of vaccinees. Five volunteer protocols were carried out on VEE (2), Rocky Mountain spotted fever and influenza (2).						
Publication: Arthropod-borne Virus Information Exchange 30:137-140, 1976.						
Available to contractors upon originator's approval.						

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 002: Evaluation of Experimental Vaccines in Man in BW Defense Research

Background:

Research under this work unit has continued with administration of selective vaccines to at-risk laboratory workers, clinical and serologic observation of laboratory workers and limited trials using volunteers from the USAMRIID professional staff. Protocols have been submitted to the Army Investigational Drug Review Board (AIDRB) for future testing of new vaccines. Since the termination of the draft, Project Whitecoat has been severely curtailed, thus limiting the number of volunteers available.

Progress:

Summaries of reports to AIDRB follow:

LVS Tularemia Vaccine. During the reporting period ending November 1975, 82 individuals received the vaccine, 38 primary, and 11 booster inoculations at USAMRIID; 33 booster inoculations were given at Dugway Proving Grounds. Two primary vaccinees experienced a reaction characterized by adenopathy, fever, arthralgias and malaise of one day's duration. There were 4 local reactions characterized by a 1-cm papule at the site of inoculation. Serologic post-vaccination titers are available on 38 of the subjects receiving primary vaccination; 5% had reciprocal titers of < 10, 10% had 20-40, 31% had 80-160, 31% had 320-640, and 21% had 1280-5120.

WEE Vaccine, Inactivated, Dried (Lot 2-1974). Use of this vaccine has been approved by the Army Investigational Drug Review Board. It has not yet been utilized.

Q Fever Vaccine (Henzerling Strain). During the reporting period ending 23 December 1975 for this vaccine, 40 individuals received a primary series of 3 vaccination doses, 39 which were given at USAMRIID. No secondary immunizations were administered. Two individuals experienced local reaction consisting of tenderness and swelling at the site of injection after the second shot of the series; there were no systemic reactions. One individual developed a 2-cm area of induration approximately 3 wk after the third injection. This resolved spontaneously over a period of several months.

One individual with preexisting antibody titer at 1:8 showed an increase to 1:64 by complement fixation (CF). All other individuals remained negative by CF.

EEE Vaccine, NDBR 104. For the last reporting period, 80 subjects received the primary immunizing series. There were no local or systemic reactions among them; 84% of 43 individuals tested showed had detectable HI antibody 1 month after the second injection. Intradermal boosters were given to 81 individuals during the last reporting period. There were 2 minor local reactions, <2 cm in diameter. Serologic data on 77 individuals showed that this represents a 2-fold increase over prebooster values in 48%.

VEE Vaccine, Live, Attenuated, NDBR 102. During the reporting period 77 immunizations were given at Dugway Proving Grounds and 64 at USAMRIID, using 2 different lots of vaccine. Ten individuals experienced systemic reactions 5-9 days after immunization, characterized by chills, fever, headache, arthralgias and diarrhea. No individuals were hospitalized. All individuals were seronegative prior to immunization; serologic data on 62 of the vaccinees showed that 56% remained seronegative and 35% had HI titers of 1:20 and 8% had titers of 1:40.

WEE Vaccine, MNLBR 106. During the reporting period 129 individuals received this vaccine, 66 as booster ID inoculations and 63 as primary SC inoculations; 64 of the boosters and 37 of the primaries were given at USAMRIID. No local or systemic reactions were noted. Of 64 individuals receiving the booster immunization, 5% had a 4-fold rise in titer, 31% had a 2-fold rise in titer; 4 of 6 individuals negative prior to the booster converted to positive. Serologic data on 34 primary immunizations indicate that all persons were seronegative prior to vaccination and 24% showed no change in titer.

VEE Vaccine, Inactivated, MNLBR 109. A protocol for evaluation of this vaccine in man has received approval by AIDRB. Plans for study in the staff of USAMRIID have been made.

A number of volunteer protocols (AIDRB approved) were conducted.

Persistence of Venezuelan Equine Encephalomyelitis Antibodies Following Vaccination with the Live, Attenuated, TC-83/3-2 VEE Vaccine (Medical Division Protocol 75-3). This study was initiated and completed this year. Data concerning the persistence of neutralizing antibodies 5-6 yr after vaccination with TC-83 VEE vaccine has been published¹ but the population studies were exposed repeatedly to Group A arboviruses, thus rendering the data inconclusive. The purpose of this project was to determine the persistence of antibodies to viruses of the VEE complex after a single, defined antigenic exposure to VEE virus in the form of TC-83 VEE vaccine. Twenty project

Whitecoat personnel who had had a single defined antigenic exposure to VEE virus were bled 7 and 9 yr after that exposure to TC-83 vaccine. Assessment of the antibody titers in these individuals provide the most accurate reflection of the long term persistence of antibodies to VEE virus following vaccination with the TC-83/3-2 vaccine. The serological results are shown in Table I.

TABLE I. SEROLOGICAL CROSS-REACTIONS AMONG VEE VIRUS SUBTYPES IN VOLUNTEERS 7 AND 9 YEARS AFTER TC-83/3-2 VEE VACCINE

Virus Subtype	Positive ^a		Reciprocal Geom. Mean Titer ^b
	No./Total	%	
<u>7 years</u>			
IA (TC-83)	5/5	100	121
(Trinidad)	5/5	100	26
IB (MF-8)	4/4	100	56
IC (V-198)	4/4	100	135
ID (3880)	2/4	50	14
IE (Mena II)	1/4	25	20
II (Fe-3-7c)	3/4	75	25
III (Mucambo)	0/4	0	0
IV (Pixuna)	0/4	0	0
<u>9 years</u>			
IA (TC-83)	15/15	100	163
(Trinidad)	14/15	93	38
IB (MF-8)	14/15	93	100
IC (V-198)	15/15	100	191
ID (3880)	6/15	40	18
IE (Mena II)	4/15	27	24
II (Fe-3-7c)	12/15	80	54
III (Mucambo)	2/15	13	28
IV (Pixuna)	1/15	7	10

a. PRN₈₀ ≥ 1:10

b. Of all titers ≥ 1:10

Neutralizing antibody in titers greater than 1:10 existed in 19 of 20 inoculated volunteers. This antibody was directed against the homologous TC-83 strain and antigenically related epizootic subtypes, IA Trinidad, IB, and IC. Less antibody was detected in the sera directed against more antigenically distant enzootic serotypes, ID, IE, II, III, and IV. No antibody was detected against EEE or WEE viruses, and no Group A arbovirus antibody was detected in 4 sham-vaccinated volunteers injected with vaccine buffer 7 years ago (not shown in Table I). We conclude that the TC-83/3-2 vaccine induced VEE epizootic antibody in 95% of these persons, and that the antibody persisted for at least 7-9 years. Fewer volunteers had antibody to enzootic compared to epizootic subtypes, which suggests that the vaccine may provide less complete protection against the enzootic strains.

The antigenically restricted nature of the serological response after TC-83 vaccine was confirmed in another at-risk group of 10 Group A arbovirus-negative laboratory personnel (Table II). These 10 persons received TC-83 (NDBR 102, run 4, lot 2) vaccine and were bled 28 days later. The number of antibody converters and geometric mean titers were higher against the epizootic VEE subtypes IA, IB and IC than against the enzootic virus subtypes. Comparison of antibody titers at 1 month (Table II) with those at 7-9 years (Table I) reveals that the titers against the epizootic strains tend to persist, while those against the enzootic strains tend to fall more during the convalescent years.

In the third study, 9 laboratory workers, previously vaccinated with VEE, EEE, and WEE viruses were routinely boosted with EEE and WEE vaccines. Their serological responses were measured 2-8 wk later in an attempt to determine if these inactivated vaccines could boost heterologous antibody titers to VEE virus subtypes. The results (Table III) indicate that only 1 or 2 persons developed antibody to any VEE virus subtype after heterologous vaccines. A significant boost in titer occurred against the homologous viruses WEE and EEE, but not against VEE subtypes, except for types IE and III. These results suggest that booster vaccination with EEE and WEE vaccines have only a minimal effect, if any, on VEE antibody titers.

We were provided the opportunity to examine the diagnostic specificity of the plaque reduction neutralization test (PRNT) after a mild febrile illness presumably caused by a laboratory-acquired VEE infection. This person had been vaccinated 3 years previously with TC-83. The infecting VEE strain was unknown; many VEE subtypes were being used at the time of illness, but no virus isolation was attempted from the patient. Table IV shows the titers of neutralizing antibody in sera taken during illness, and 3 and 15 months later. During illness epizootic antibody titers (IA, IB, IC) were higher than enzootic titers, as previously shown in Tables I and II. During convalescence there was a broad anamnestic antibody response to all VEE subtypes, but not to WEE or EEE viruses, rendering it impossible to identify the infecting VEE strain or strains by serological methods.

TABLE II. SEROLOGICAL CROSS-REACTIONS AMONG VEE VIRUS SUBTYPES IN 10 PERSONS VACCINATED WITH TC-83 (NDBR 102) VEE VACCINE

Virus Subtype	No. Positive/10	Reciprocal GMT ^a
IA (TC-83)	10	422
(Trinidad)	10	70
IB (MF-8)	9	186
IC (V-198)	10	845
ID (3880)	8	48
IE (Mena II)	6	90
II (Fe-3-7c)	8	113
III (Mucambo)	3	20
IV (Pixuna)	2	28

a. $\geq 1:10$ by PRN₈₀ at 28 days.

TABLE III. SEROLOGICAL CROSS-REACTIONS AMONG 9 VEE SUBTYPES, AND WEE AND EEE AFTER 9 IMMUNE^a PERSONS WERE BOOSTED WITH WEE - EEE VACCINES

Virus Subtype	Prebooster		Postbooster	
	No. Positive ^b	GMT	No. Positive ^b	GMT ^c
VEE				
I (TC-83)	4	191	5	209
(Trinidad)	5	39	6	50
IB (MF-8)	5	80	6	100
IC (V-198)	6	224	7	290
ID (3880)	4	20	5	30
IE (Mena II)	0	<10	2	100
II (Fe-3-7c)	4	<17	5	30
III (Mucambo)	0	<10	2	20
IV (Pixuna)	0	<10	1	10
WEE	6	24	8	174
EEE	6	32	9	214

a. Previously vaccinated with VEE, EEE and WEE vaccines.

b. $\geq 1:10$ by PRN₈₀

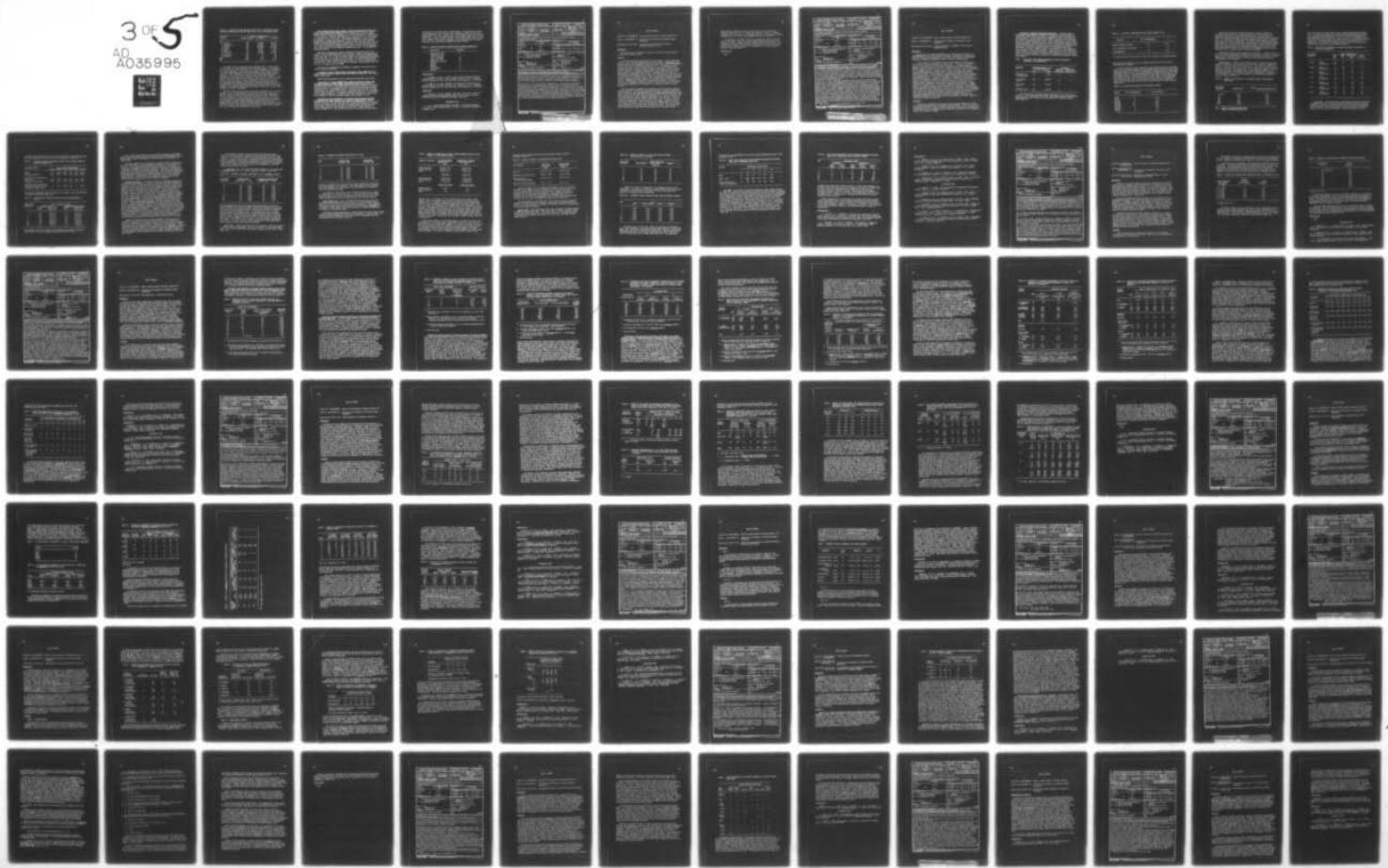
c. Of responders.

AD-A035 995 ARMY MEDICAL RESEARCH INST OF INFECTIOUS DISEASES FR--ETC F/G 6/5
ANNUAL PROGRESS REPORT - FY 1976.(U)
JUL 76 F B ABELES, A O ANDERSON, J B ARENSMAN

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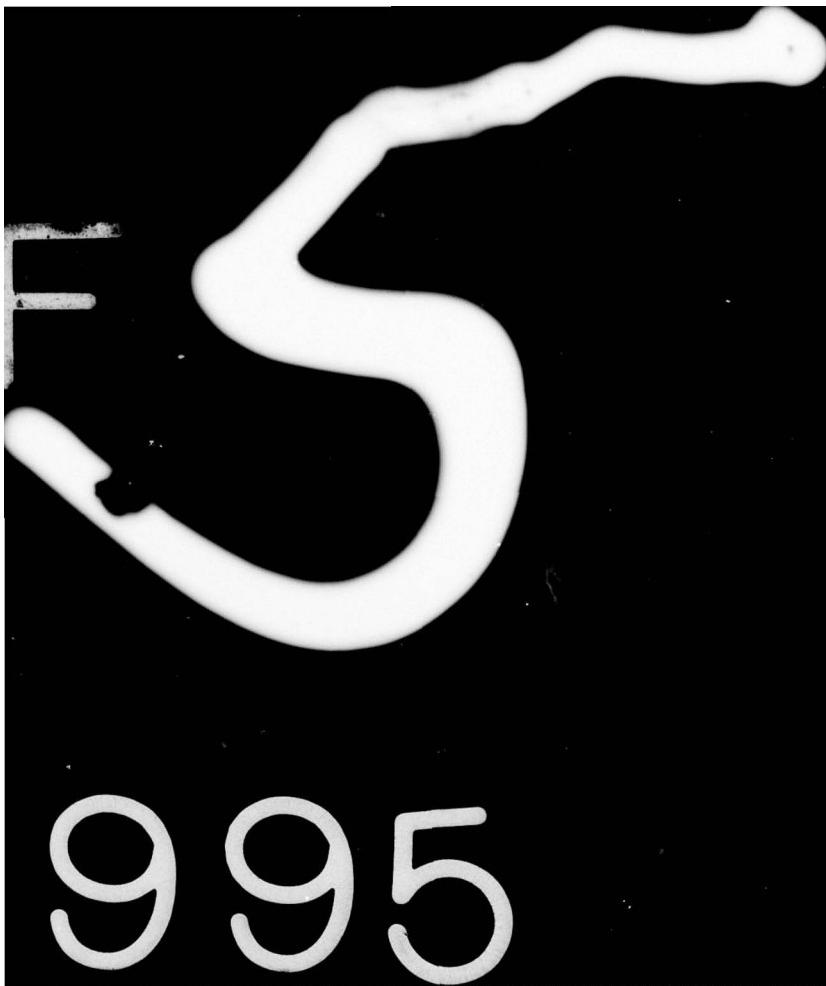


TABLE IV. SEROLOGICAL CROSS-REACTIONS AMONG GROUP A ARBOVIRUSES AFTER A PRESUMED LABORATORY-ACQUIRED VEE INFECTION IN A TC-83-VACCINATED PERSON

Virus and Subtype		Reciprocal PRN ₈₀ Titer		
	During Illness	3 months	14 months	
VEE				
IA (TC-83)	160	327,680	163,840	
(Trinidad)	80	20,480	10,240	
IB (MF-8)	80	163,840	81,920	
IC (V-198)	320	327,680	655,360	
ID (3880)	10	10,240	640	
IE (Mena II)	10	20,480	10,240	
II (Fe-3-7c)	40	10,240	10,240	
III (Mucambo)	<10	320	80	
IV (Pixuna)	<10	40	20	
WEE	80	40	40	
EEE	1280	1,280	2,560	

In another study, we obtained throat washings from 14 at-risk laboratory personnel routinely immunized with TC-83 VEE vaccine. The purpose was to determine if VEE virus recovery is related temporally to development of clinical symptoms. This question was raised by the recovery of VEE virus from throat washings of 2 vaccinees during vaccine-related febrile reactions. The isolates from these 2 persons killed 70% of hamsters, whereas the TC-83 virus strain is known to kill only 10% of hamsters. The possibility is thus raised that TC-83 may either revert to a more virulent strain in some vaccinees, or that a few virulent virions exist in the vaccine inoculum and emerge in some persons to cause febrile reactions. The possibility that vaccine reactions are caused by virulent VEE can be excluded if (1) vaccine nonreactors also harbor virulent VEE in their nasopharynx, or (2) VEE strains isolated from vaccine reactors do not all show increased virulence for animals.

Thus far we have recovered virus from throat washings of 6 to 7 vaccine reactors. In 5 of these persons the isolations were made during mild febrile illness, occurring in most reactors 9-11 days after immunization. The virus isolates have been tentatively identified as VEE. We did not recover virus from 7 vaccine nonreactors who were cultured for 8-13 days out of the first 14 days postvaccination, including the critical 9-11-day postvaccination period. These preliminary results indicate that the emergence of recoverable virus in the nasopharynx is associated with febrile vaccine reactions. The virulence testing of throat wash isolates is pending. More vaccinees will be studied in the future.

Rocky Mountain Spotted Fever Vaccine, Formalin-Inactivated, Sheila Smith Strain, Chick Embryo Cell Origin (Medical Division Protocol 76-1). This vaccine has been tested in 6 members of the professional staff of USAMRIID. A full course of vaccination consisted of 3 SC 0.5-ml doses of a 1:10 dilution at 1-wk intervals. Four of the 6 individuals completed the full course without systemic or local reactions. One volunteer developed a local reaction consisting of erythema and swelling <1 cm 24 hr after the first injection. Another volunteer developed areas of erythema (~2 cm) and induration (~8 mm) 24 hr after receiving the second injection; these reactions were noted both at the site of the second injection and at the site of the initial administration. This was felt to be a possible Jones-Mote reaction. It decreased in intensity by 48 hr.

Immunologic response data indicate significant immunogenicity of this vaccine. In one individual receiving the complete course, microagglutination titers rose from 1:4 to 1:64 at 56 days; indirect fluorescence antibody (IFA) titers rose from 1:8 to 1:16 at 24 days; lymphocyte transformation stimulation ratio rose from 2 at -35 days to 29 at +56 days. In another individual who received only 2 doses, the MA titer rose from 1:4 to 1:16 at 35 days and the IFA rose from 1:8 to 1:32 at 35 days; the lymphocyte transformation ratio rose from 7 at -14 days to 24 at +35 days.

Evaluation of the serologic and lymphocyte response in the other volunteers is currently underway.

Acceptability Study of VEE Vaccine, Inactivated, Dried, MNLBR 109, Lot C-84-1 (Medical Division Protocol 76-2) was written and approved. The project began in April 1976.

Medical Division Protocol 76-3 is reported under Work Unit 834 02 003.

Immunization of At Risk USAMRDC (Fort Detrick) Laboratory Workers with Monovalent Influenza A/Swine (A/New Jersey/8/76) Virus Vaccine (Medical Division Protocol 76-4). On 18 May 1976, 114 laboratory personnel at-risk to swine influenza virus, A/New Jersey/76 infection, were inoculated with swine influenza vaccine. This subunit vaccine, containing 400 CCA units of virus antigen/dose, was injected IM (0.05 ml). Reactions noted for the 2 following days are shown in Table V. No placebo inoculations were given. On the basis of the number and severity of systemic and local reactions, the vaccine was well tolerated. Blood for antibody response will be drawn at 3 wk.

Reactogenicity and Antigenicity of Influenza Virus Vaccines: Bivalent A/Victoria/75 and A/New Jersey/76 and Monovalent B/Hong Kong/72 (Medical Division Protocol 76-5) was initiated in May. Approximately 175 civilian and military volunteers from the Fort Detrick community participated in a double-blind study. Each volunteer received (1) placebo alone, (2) 500 CCA unit dose of B/Hong Kong/72, (3) 400 CCA of A/Victoria/75 + A/New Jersey/76 ("Swine Influenza"), or both the 800 CCA unit dose of A/Victoria and A/Swine

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in addition to the 500 CCA dose of B/Hong Kong. The vaccines were inactivated preparations; the study has been stratified so that vaccines from 4 manufacturers were used. Three of the 4 A/New Jersey (Swine) vaccines were recombinants. Two of the manufacturers have produced whole virus vaccines while 2 have produced subunit vaccines. In this study there were no immediate reactions. There was an incidence of less than 5% of mild systemic reactions, including malaise, myalgia and fever of <101°.

TABLE V. SIGNS AND SYMPTOMS FOLLOWING SWINE INFLUENZA IMMUNIZATION

Observation	% Response
Temperature >100F	<1
Inoculation site	
Pain or burning	2
Mild tenderness	20
Redness (< 5 mm)	13
Induration (< 5 mm)	17
Malaise	7
Myalgia	4
Headache	11
Feverishness	3
Nausea	4

Presentations:

1. Edelman, R. and D. S. Burke. The attenuated Venezuelan equine encephalomyelitis vaccines: their antigenic potency in man. Presented, National Meeting, Am. Soc. Trop. Med. Hyg., New Orleans, LA, 14 Nov 1975.

2. Burke, D. S. and R. Edelman. The attenuated Venezuelan equine encephalomyelitis vaccines: limitations of their use in man. Presented, National Meeting, Am. Soc. Trop. Med. Hyg., New Orleans, LA, 14 Nov 1975.

Publication:

1. Burke, D. S. and R. Edelman. Mar 1976. The use of attenuated Venezuelan equine encephalomyelitis (VEE) vaccines in man: A review. Arthropod-Borne Virus Information Exchange, 30:137-140.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 Jul 1970. Annual Progress Report, FY 1970. p. 114-115, Fort Detrick, Frederick, MD.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION# DA OB6411	2. DATE OF SUMMARY 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUMRY 76 01 19	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SECY# U	6. WORK SECURITY# U	7. REGRADING# NA	8. DOD/NIN INSTRN# NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: a. PRIMARY 62760A	PROGRAM ELEMENT	PROJECT NUMBER 3A762760A834		TASK AREA NUMBER 02	11. LEVEL OF SUM A. WORK UNIT 003	
b. CONTRIBUTING	c. CONFIDENTIALITY CARDS 114(e)(f)					
12. TITLE (Punctuate with Security Classification Code) (U) Evaluation of prophylaxis and therapy of infectious diseases in man						
13. SCIENTIFIC AND TECHNOLOGICAL AREAS 003500 Clinical medicine; 004900 Defense; 003200 BW, CW, RW						
14. START DATE 62 09	15. ESTIMATED COMPLETION DATE CONT	16. FUNDING AGENCY DA	17. PERFORMANCE METHOD C. In-house			
18. CONTRACT/GRAANT		19. RESOURCES ESTIMATE				
a. DATES/EFFECTIVE: EXPIRATION: b. NUMBER: NA		FISCAL YEAR	PREVIOUS 76	20. PROFESSIONAL MAN YRS 0	21. FUNDS (in thousands) 0	
c. TYPE: d. AMOUNT: e. CUM. AMT.		CURRENT	77	0	0	
22. RESPONSIBLE DOD ORGANIZATION		23. PERFORMING ORGANIZATION				
NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		NAME: Medical Division USAMRIID ADDRESS: Fort Detrick, MD 21701				
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Punish blank if U.S. Academic Institution) NAME: Ascher, M. S. TELEPHONE: 301 663-7361 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: Harber, P. H.				
24. GENERAL USE Foreign intelligence considered		POC:DA				
25. REFERENCES (Punctuate each with Security Classification Code) (U) BW defense; (U) Military medicine; (U) Prophylaxis; (U) Therapy; (U) Infectious diseases; (U) Volunteers; (U) Malaria						
26. TECHNICAL OBJECTIVE, 26. APPROACH, 26. PROGRESS (Punctuate individual paragraphs identified by number. Punctuate each with Security Classification Code.) 23 (U) Assess the effect of antimicrobials and various drug regimens in key infectious diseases. This work unit is an essential element in a comprehensive program for medical defense against BW agents and other infections of unique military importance by allowing the initial testing of new drugs for chemoprophylaxis or therapy. 24 (U) Various drugs are tested in volunteers under strict protocol conditions.						
25 (U) 75 07 - 76 06 - One study was undertaken this year entitled "Rejuvenation and preservation of Plasmodium vivax (Chesson strain) and assessment of blood schizontocidal activity of mefloquine hydrochloride." A single volunteer received infectious blood; he had a single typical paroxysm of malaria on day 15. His blood was then obtained for cryopreservation at the peak of parasitemia. Therapy was given in the form of mefloquine. The individual was free of parasites after 3 days and is currently under observation for possible recrudescence of infection. Thus, the Chesson strain of malaria has been successfully and safely rejuvenated. This result has allowed this valuable model strain to remain available for additional study and use in the continuing development of antimalarial drugs in man.						

Available in consecutive even numbers.

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PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65
AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

* U.S. GPO: 1974-840-843/0691

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 003: Evaluation of Prophylaxis and Therapy of Infectious Diseases in Man

Background:

The work unit was established to assess the effect of antimicrobials in infectious diseases in man.

Progress:

In collaboration with investigators from the WRAIR, a project was undertaken this year entitled, Rejuvenation and preservation of Plasmodium vivax (Chesson strain) and assessment of blood schizontocidal activity of mefloquine hydrochloride (Medical Division Protocol No. 76-3). The Chesson strain of P. vivax was first isolated during World War II and is characterized by a series of relapses occurring at short intervals following a primary mosquito induced infection. Because of this property it has remained for more than thirty years the most important strain of its species for the study and development of antimalarial drugs in man. The availability of this well characterized strain is essential to the future of the Army antimalarial program. In early 1976 it was realized that a single cryopreserved specimen of Chesson strain P. vivax was all that remained in a suitable form for human use. Because of considerable uncertainty concerning the viability of these parasites, it was considered urgent to attempt to preserve the Chesson strain by an additional passage in man followed by its long-term storage in liquid nitrogen.

After the recently standardized procedures for human volunteer studies had been followed in detail and clearances obtained, a single volunteer was inoculated with 5 ml of resuspended cells containing approximately 5×10^6 parasites. Thick and thin smears for malaria were examined daily beginning 5 days after inoculation; all were negative through day 14. On day 15 slight fever and diaphoresis were noted. Malaria smears were still negative. On day 15 a typical malarial paroxysm occurred with chills, fever of 101.6F, headache and profuse diaphoresis. Malaria parasites were seen; a quantitative count of $90/\text{mm}^3$ was obtained. On day 16 the count was $200/\text{mm}^3$; 200 ml of blood were withdrawn from the subject and transported to WRAIR where it was immediately glycerolized and quick-frozen in liquid nitrogen. The volunteer was then treated with a single dose of mefloquine with no adverse effects.

Malaria smears remained positive on day 16 and 17 and a second mild paroxysm occurred on day 17. Smears were negative on day 18. Parasite clearance time was 66 hr; fever remission was 61 hr. There were no adverse changes noted in any laboratory parameters during this infection.

In conclusion, the Chesson strain of P. vivax was safely and successfully rejuvenated. Freshly isolated specimens have been stored in 2-ml aliquots in liquid nitrogen and should remain viable for many years. The experimental drug mefloquine was well tolerated; its efficacy as a blood schizontocide against P. vivax will be determined in further follow-up studies of this individual.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION#	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
3. DATE PREV SURVEY	4. KIND OF SURVEY	5. SUMMARY SECY*	6. WORK SECURITY*	DA OF6415	76 07 01	DD-DR&E(AR)636	
75 07 01	D. CHANGE	U	U	NA	NL	DA SPECIFIC DATA- <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	D. LEVEL OF SURVEY A. WORK UNIT
10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	62760A	3A762760A834		02	013		
b. CONTRIBUTING							
c. /d. /e. /f.	CARDS 114(e)(f)						
11. TITLE (Prefix each with Security Classification Code)* (U) Cellular phenomena in lymphatic tissues during immune responses							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS* 003500 Clinical medicine; 004900 Defense; 012600 Pharmacology							
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NAME: Metzger, J. F.	TELEPHONE: 301 663-2833	NAME: Anderson, A. O.	TELEPHONE: 301 663-7211	ASSOCIATE INVESTIGATORS			
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24. TECHNICAL OBJECTIVE,* 25. APPROACH, 26. PROGRESS (Prefix each paragraph identified by number. Prefix text of each with Security Classification Code)*							
23. (U) Define regulatory mechanisms of lymphocyte recirculation and cellular interaction in tissues during the in vivo immune response; use this information to develop new and more potent adjuvants for use with military vaccines; materials developed in this manner should significantly hasten the interval between vaccination and protection							
24. (U) Use morphological and rabiolabeled indicators of lymphocyte kinetics to study factors influencing lymphocyte traffic into lymph nodes. Success will permit testing of materials for adjuvant activity.							
25. (U) 75 07 - 76 06 - Lymphocyte emigration across the walls of lymph node HEV depends upon (1) trypsin and EDTA-sensitive glycoprotein receptors on the surface of lymphocyte microvilli and complementary receptors on HEV endothelial cells; and the ability: (2) of the lymphocyte to actively migrate using cytochalasin-A sensitive microfilaments; (3) to show oriented movement because of colchicine-sensitive microtubules; and (4) to sense a chemotactic gradient which is formed through the unique permeability characteristics of HEV. Adjuvants achieve their effects by increasing influx and retention of recirculating lymphocytes in the lymph node paracortex early in the primary immune response; inevitably exposing more potentially immunoreactive cells to antigen. Adjuvants enhance proliferation and maturation of potential antibody-forming cells, while maintaining prolonged influx of accessory cells, and activate macrophages which may facilitate antigen processing. Preliminary findings suggest that both human dialysable transfer factor and stabilized poly I:C may be useful adjuvants for the immune response against killed Venezuelan equine encephalomyelitis virus.							
Publications: Am. J. Pathol. 80:387-418, 81:131-160, 1975. Immunology 31:455-473, in press, 1976.							

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BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 013: Cellular Phenomena in Lymphatic Tissues During Immune Responses

Background:

Recirculation by long-lived lymphocytes provides constant traffic of immunocompetent cells across lymphatic tissues. Lymphocytes emigrating from the blood through high endothelial venules (HEV) are exposed to concentrated antigen and endogenous lymphokines within the lymph node parenchyma.

In the rat about 10^9 lymphocytes pass through lymphatic tissues every 24 hr as determined by thoracic duct cannulation.¹ This traffic provides for clonal selection which is necessary for induction of an immune response. Because of the heterogeneity of *a priori* antigen-reactive cells, approximately 10^4 - 10^5 lymphocytes must be exposed to antigen in order for 1 cell to proliferate into an antigen-specific reactive clone. A 20-mg rat lymph node normally contains 10^8 lymphocytes and maintains this number through daily flux of recirculating cells. This dynamically changing population of immunocompetent cells represents the critical mass necessary for normal protection from day-to-day antigenic insults. It would be desirable to manipulate this cellular traffic to increase the magnitude and rapidity of a protective immune response. However, this response can not be regarded simply as the result of interactions between individual lymphocytes and the appropriate antigen. Recent studies have established that successful immunization depends upon a complex sequence of antigen-binding, antigen presentation and cellular collaboration between thymus-derived lymphocytes, marrow-derived lymphocytes, and macrophages. It is likely that these interactions take place in or near HEV, since lymphocytes selectively adhere and actively migrate across HEV walls and immunoblasts are first seen in the immediate perimeter of HEV after immunization. It is becoming increasingly evident that hemodynamic changes in the lymph node vasculature during the early immune response may markedly influence lymphocyte traffic and cellular cooperation.²

Progress:

Studies were conducted in collaboration with Contract DAMD-17-74-C-4095, and in-house with MAJ Ascher, CPT Andron, and LTC Houston. In vivo and in vitro techniques were used to study the basic mechanisms of lymphocyte recirculation and potentiation of the immune response produced by agents which alter lymphocyte traffic into lymphatic tissues.

Mechanism of lymphocyte homing and emigration. Sequential events during lymphocyte emigration from HEV were studied by scanning (SEM) and transmission (TEM) electron microscopy, regional perfusion techniques and autoradiography. Results indicate that lymphocytes selectively adhere to HEV surfaces through microvilli which attach to shallow pits on the luminal surfaces of venular endothelial cells. These intercellular contact points resist hydrodynamic shearing forces imposed by intraarterial perfusion with 10% dextran-normal saline solutions, but can be disrupted by perfusion with agents which solubilize oligosaccharides from endothelial glycocalyx (0.9% NaCl), hydrolyze lymphocyte surface glycoproteins (0.1% trypsin), or chelate divalent cations (0.01 M EDTA). Data produced by these perfusion-flush studies of the isolated mesenteric lymph node are in the form of cell output in effluent perfusate collected from the mesenteric vein and morphologic studies after alcian blue perfusion to demonstrate endothelial surface coat (Table I).

TABLE I. CELL OUTPUT FROM PERFUSED MESENTERIC NODES AND LYMPHOCYTE ADHERENCE TO HEV ENDOTHELIUM

PERFUSATE	LYMPHOCYTE OUTPUT $\times 10^3$		HISTOLOGY	
	Mean	1 min Collection Range	Adherent Lymphocytes	Endothelial Surface Coat
10% dextran in 0.9% NaCl (DS)	35	30-40	+	+
0.1% trypsin in DS	254	190-340	0	+
0.01 M EDTA in DS	137	70-190	0	+
0.9% NaCl alone	184	140-240	0	0

After this initial attachment phase, lymphocytes enter apical clefts between 2 or more endothelial cells where they assume a motile configuration characterized by loss of microvilli and formation of irregular surface folds (Table II).

TABLE II. LOCATION OF LYMPHOCYTES ON HEV LUMINAL SURFACE BY SEM

LOCATION	NO. OF LYMPHOCYTES	%
Within endothelial clefts	129	83.3
Over endothelial cell centers	5	3.2
Indeterminable ^a	21	13.5

^a Precise location was obscured by other cells attached to the luminal surface.

These migrating lymphocytes show cytoplasmic polarity which is oriented in the direction of movement.

In vitro studies of motile lymphocytes have shown that these cells migrate forward with the nucleus leading and the organelle rich uropod following. This observation was used to identify the angle of cytoplasmic polarity of migrating lymphocytes fixed *in situ*. Electron micrographs of transverse sections of HEV were studied and each intramural lymphocyte was assigned an angle based on its cytoplasmic polarity. The reference point was the center of the vessel where 180° pointed out and 0/360° pointed into the lumen. Most of the lymphocytes were oriented within the sector 161-200° which pointed from the HEV lumen toward the node parenchyma, and 89.7% were located within the sectors 121-240° (Table III).

TABLE III. DIRECTION OF MIGRATION BY CYTOPLASMIC POLARITY

SECTOR	NO. OF LYMPHOCYTES	% IN EACH SECTOR
0-40°	2	1.5
41-80°	2	1.5
81-120°	4	2.9
121-160	36	26.8
161-200	69	51.0
201-240°	16	11.9
241-280	1	0.7
281-320	5	3.7
321-360	0	0.0

Since passive migration across the HEV wall within an endothelial cell vacuole should result in random distribution of cell polarity, the nonrandom orientation of emigrating lymphocytes was further evidence in support of our contention that these cells actively migrate between endothelial cells. In addition, cytoplasmic polarity suggested the presence of a motive force such as a chemotactic gradient. Previous studies³ using macromolecular extracellular tracers localized migrating lymphocytes in extracellular compartments which were contiguous with the vascular lumen. Similar tracer studies were used to characterize the unidirectional permeability of the HEV wall.⁴ These observations supported our hypothesis that lymphocytes migrate between endothelial cells oriented toward a chemotactic gradient.

Cytoplasmic polarity and cell motility are related to alterations in the state of cytoplasmic actomyosin filaments and microtubular proteins. We studied the effects of cytochalasin and colchicine on lymphocyte recirculation since these materials have been shown to solubilize actin proteins and prevent polymerization of microtubules.

Cytochalasin A. Thoracic duct lymphocytes (TDL) collected from Bollman fistulae in Lewis rats were incubated in ³H-uridine, washed and incubated for 30 min in a solution containing cytochalasin A. These cells (4×10^8) were infused IV into each Lewis rat and the axillary nodes were removed at selected times for histology and autoradiography. Total lymphocyte migration indices (LMI) (labeled cells + nonlabeled cells) were tabulated for HEV in rats receiving normal TDL cells and TDL treated with cytochalasin A. Addition of 4×10^8 normal TDL causes a modest increase in the LMI which subsequently returns to normal (Table IV).

TABLE IV. EFFECTS OF CYTOCHALASIN A ON LYMPHOCYTE ENTRY INTO PERIPHERAL LYMPH NODES

TIME AFTER INJECTION	LMI ^a	
	Normal Cells	Cytochalasin-Treated Cells
0 min	0.75	0.75
10	1.00	1.25
30	1.35	1.90
60	1.25	1.85
120	1.12	1.75
180	1.00	1.60
240	0.82	1.40

^aLMI = $\frac{\text{number of migrating lymphocytes}}{\text{number of high endothelial cells}}$

The total LMI for the lymph nodes, in rats receiving cytochalasin-treated cells, is markedly increased at 30 min and remains elevated for the ensuing 6 hr. This apparently paradoxical effect was resolved on examination of autoradiographs of these specimens. The rise in the total LMI reported in Table IV was primarily related to increased migration of nonlabeled endogenous lymphocytes. Autoradiographs indicated that the cytochalasin-treated lymphocytes were retained in HEV lumens while normal cells rapidly crossed the HEV wall to enter the node parenchyma (Table V).

TABLE V. DISTRIBUTION OF NORMAL AND CYTOCHALASIN-TREATED LYMPHOCYTES AT THE SITE OF EMIGRATION

TIME AFTER INJECTION		% CELLS LOCALIZED TO:			
		HEV lumen	HEV wall	Perivenular space	Node parenchyma
10 min	Normal	22	41	30	7
	Cytochalasin	100	--	--	-
30 min	Normal	7	20	37	36
	Cytochalasin	82	14	3	1
1 hr	Normal	0	3	20	77
	Cytochalasin	81	13	5	2
2 hr	Normal	6	8	19	67
	Cytochalasin	68	27	3	3
4 hr	Normal	9	7	13	71
	Cytochalasin	31	7	52	10
12 hr	Normal	14	9	23	54
	Cytochalasin	8	8	50	35
24 hr	Normal	10	10	14	66
	Cytochalasin	2	2	51	45

Colchicine. Colchicine prevents polymerization of tubulin; therefore, treatment of cells with colchicine should ablate cytoplasmic orientation, movement of organelles, and secretion which are cell functions dependent on microtubules. Colchicine was used *in vivo* and *in vitro* to assess its effect on recirculation of lymphocytes. After IV infusion of 0.1 mg

colchicine the total LMI fell from 0.75 to 0.29 within 3 min after injection. The LMI remained near this level for the remaining 24 hr (Table VI).

TABLE VI. HISTOLOGIC SCORING OF LYMPHOCYTE ENTRY INTO PERIPHERAL NODES OF COLCHICINE-TREATED RATS

TREATMENT GROUPS	LMI AT TIMES POSTTREATMENT					
	3 min	30 min	1 hr	4 hr	6 hr	24 hr
Normal	0.75	0.80	0.76	0.79	0.81	0.80
Colchicine-treated	0.56	0.25	0.25	0.14	0.12	0.08
4x10 ⁸ TDL cells infused into						
Normal rats	1.09	1.36	1.25	0.87	0.69	0.80
Colchicine-treated rats	0.29	0.22	0.28	0.10	0.11	0.10
4x10 ⁸ TDL cells treated with						
colchicine in vitro and then	1.14	1.23	0.80	0.78	--	0.89
infused into normal rats						

The output of lymphocytes from the thoracic duct began to fall precipitously 4 hr after drug infusion (Table VII).

TABLE VII. THORACIC DUCT LYMPHOCYTE OUTPUT IN COLCHICINE-TREATED RATS
(N = 5-7)

HOURS AFTER INJECTION	HOURLY LYMPHOCYTE OUTPUT			
	NORMAL RATS		COLCHICINE-TREATED RATS	
	Volume ml	Total (10 ⁶) mean \pm SD	Volume ml	Total (10 ⁶) mean \pm SD
1	3.1	26.6 \pm 4	2.7	37.8 \pm 9
2	3.0	27.2 \pm 7	2.2	27.5 \pm 8
3	3.2	27.4 \pm 6	2.1	22.2 \pm 6
4	2.4	26.5 \pm 4	2.0	11.3 \pm 3
5	2.6	25.8 \pm 5	2.0	11.8 \pm 3
20	3.0	19.6 \pm 6	1.5	8.2 \pm 2
24	2.1	18.9 \pm 6	2.1	8.1 \pm 2
48	1.9	17.8 \pm 4	1.5	2.5 \pm 0.3

This delayed fall in cell output can be explained as related to the mean transit time of recently emigrated lymphocytes which is 5-6 hr.

Cells treated with colchicine *in vitro* appear to migrate across HEV. However, the rate of migration as determined by the LMI is modestly reduced when compared to LMI's from animals receiving similar concentrations of normal cells.

These data suggest that colchicine has a greater effect on migration *in vivo* possibly due to its heterogeneous activities on cell structure and secretion. It is speculated that colchicine prevention of secretion of "chemotactic" substances, or surface receptors essential for "homing," may be the mode of action of IV-infused colchicine. Colchicine only nominally prevents *in vitro* treated cells from migrating. The cytochalasin experiments showed that contractile actin filaments are essential to active migration across HEV walls. Accumulations of cytochalasin-treated cells along the luminal surfaces of HEV indicated that lymphocyte homing receptors were retained despite disruption of the cell's motile apparatus.

Adjuvant experiments. The recent explosion of knowledge regarding the immune system and the current concern for increasing the weak antigenicity of tumor and viral antigens has awakened interest in the mode of action of adjuvants on the cells and tissues of the lymphatic system. Although many substances have been empirically tested for immunologic potentiation, only 4 major classes of adjuvants have proven useful. A fifth class may be evolving. Class 1 involves the incorporation of antigen in water-in-oil emulsions; Freund's adjuvant has been very effective in laboratory animals but local toxicity and its predisposition to autoimmune disease has negated its use in human beings. Class 2 including Al(OH)₃-precipitated antigens has enjoyed widespread use in pediatric immunization with diphtheria and tetanus toxoids. Class 3 mixed bacterial vaccines, BCG, and gram negative bacteria, including Bordetella pertussis, have been studied *in vivo* and *in vitro*. Their use has been limited due to febrile inflammatory sequelae. Class 4, exogenous polymers of nucleic acid, polyadenylic:polyuridylic acid (poly A:U) and polyinosinic:polycytidylic acid (poly I:C) have recently been shown to have adjuvant-like effects. Toxicity problems seem to be controlled by various stabilizing procedures. In addition, a new 5th class of adjuvant may be evolving which is water soluble, low molecular weight, nonantigenic and highly potent. In this class might belong various factors such as transfer factor (TF), prostaglandin, ubiquitin, tuftsin, thymopoietin and water-soluble glycopeptide immunoadjuvant from mycobacterial cell walls.

Despite the heterogeneity in origin and physical characteristics of adjuvants, most appear to increase antibody production by increasing the traffic of lymphocytes into lymphatic tissues, activating antigen-processing macrophages, and enhancing lymphocyte proliferation and maturation. These studies describe the response of the regional node to B. pertussis, stabilized with poly I:C lysine (PICLC) and dialyzable TF from human blood leukocytes. In other studies, PICLC and TF were used to augment the immune response to killed Venezuelan equine encephalitis virus (KVEE) (collaborative Work Unit 834 02 419).

The experimental design for these studies was relatively simple. Adjuvant or antigen-adjuvant combinations were injected SC into the right lateral thorax of Lewis rats while the contralateral side received a sham injection. A part of each group of animals was sacrificed for axillary-node weight measurement and histological examination of the regional and contralateral lymph nodes. Antigen-treated rats were transferred to LTC Houston's laboratory for sequential blood collection and measurement of TC-83 VEE plaque neutralization (PN) titers.

B. pertussis alone. The lymph node weight response to a strong antigen B. pertussis (which is also a potent adjuvant) is shown in Table VIII.

TABLE VIII. RESPONSE OF REGIONAL LYMPH NODES TO SC B. PERTUSSIS VACCINE

DAYS AFTER CHALLENGE	CONTROL NODE		B. PERTUSSIS-DRAINING NODE	
	Weight (mg)	LMI	Weight (mg)	LMI
0	18	0.75	18	0.76
1	20	0.81	45	1.48
2	19	0.80	65	1.62
3	17	0.69	61	1.24
4	18	0.76	60	1.09
7	16	0.97	41	0.86
10	18	0.84	40	0.89
14	20	0.66	41	0.80
28	19	0.82	32	0.91
35	17	0.70	22	0.73

The B. pertussis draining node enlarged rapidly and began to decline in weight after 14 days. The early spike occurred before lymphocyte proliferation and probably represented increased influx and retention of lymphocytes emigrating from the peripheral blood. The broad part of the curve most likely represents the combined effect of proliferating clones of antigen reactive cells and increased efflux of lymphocytes into the blood via the efferent lymph. These activated cells began to leave the node to seed the spleen and other lymphatic tissues after 72 hr accounting for the fall in node weight. The primary zone of reaction to strong antigens and adjuvants was located in the deep cortical thymic-dependent area of the lymph node which contains numerous high endothelial venules.

PICLC alone. Changes induced by local SC injection of PICLC were similar to those seen with B. pertussis. However, the falloff in node weight began after 7 days rather than 14 and slight weight changes were seen after 10 days (Table IX).

TABLE IX. RESPONSE OF REGIONAL LYMPH NODES TO SC PICLC

DAYS	CONTROL NODE		PICLC NODE	
	Wt (mg)	LMI	Wt (mg)	LMI
0	18	0.75	18	1.00
1	17	0.80	31	1.49
2	19	0.69	49	1.80
3	16	0.81	42	1.48
7	15	0.75	35	1.40
14	18	0.90	28	1.45
28	17	0.66	25	1.25
35	19	0.70	23	1.05

The rate of migration into these nodes was high; significant traffic was sustained throughout the experiment. Histology showed cortical expansion with small lymphocytes. Polymorphonuclear leukocytes were occasionally seen in the subcapsular sinus.

The response of the regional node to PICLC was studied over a range of doses from 4.0×10^{-4} mg to 4.0 mg. A linear increase in node weight ratios was seen from 1.4X to 2.6X between the lowest dose and 4.0×10^{-1} . Higher doses produced less node change possibly due to local toxicity.

Human leukocyte TF. Since the weight changes occurring within the first 24 hr after injection can be attributed to traffic changes and not proliferation, most of the TF studies were performed with a 25-hr endpoint. Time-course studies such as those described for PICLC and B. pertussis are in progress. These studies also include attempts to define the mechanism of action of TF on regional nodes.

Preliminary studies suggested that human dialyzable TF from random sources, produced increased lymphocyte traffic and accumulation in regional nodes. In addition, large accumulations of "activated" sinus macrophages were also seen within 24 hr of infusion (Table X).

TABLE X. EFFECTS OF HUMAN BLOOD AND CELL CULTURE TRANSFER FACTORS ON NODE WEIGHT, LMI AND MONOCYTE CONTENT

SOURCE OF DIALYSATE	TF DRAINING NODES		CONTRALATERAL CONTROLS	
	Mean Wt + SE (mg)	LMI + SE	Mean Wt + SE (mg)	LMI + SE
Human leukocyte	85.43 + 11.5		35.06 + 3.0	
WI-38 fibroblast	29.96 + 1.4		29.10 + 2.8	
BHK-21	22.90 + 6.1		9.30 + 3.8	
		LMI + SE		LMI + SE
Human leukocyte		1.22 + 0.15		0.53 + 0.10
WI-38 fibroblast		0.64 + 0.13		0.61 + 0.20
BHK-21		1.15 + 0.10		0.70 + 0.14
		Monocytes (Grade)		Monocytes (Grade)
Human leukocyte		+++		++
WI-38 fibroblast		++		++
BHK-21		+++		++

The search for a "cell-source" control revealed that baby hamster kidney (BHK-21) cells also release a factor which is capable of producing changes similar to TF. In addition, MAJ Ascher (Work Unit 834 02 417) demonstrated that all 3 factors produce comparable augmentation of proliferation in his *in vitro* assay. The response of the regional node to TF from human leukocytes appears to be dependent on the age of the rat and on the strain. Seven-month old Lewis rats show a 2.5X node increase in 24 hr while 3-month old rats have only a 1.5X increase. Mature Lewis rats show doubling in node weight while Fisher-Dunning rats show a minimal increase, and Sprague-Dawley rats show no changes at all. This is consistent with attempts to transfer skin reactivity to rats with human TF. Only Lewis rats can accept transfer of skin reactivity from human TF.⁵

Since TF is a mixture of low MW molecules it is likely that some of its contaminants (which include uracil, hypoxanthine, folic acid, and serotonin) may contribute to the node weight changes. We studied the effects of serotonin alone and serotonin blockers in combination with TF on node weight increase. If we depleted tissue serotonin (with p-chlorophenylalanine) or blocked serotoninergic receptors with methysergide, TF was incapable of causing lymph node enlargement. In contrast, exogenous serotonin had no effect on the

regional lymph node when injected in concentrations found in crude TF (60 $\mu\text{M}/\text{ml}$) (Table XI).

TABLE XI. EFFECTS OF HUMAN TF AND SEROTONIN ON NODE WEIGHT

TEST MATERIAL	TEST NODE	CONTROL NODE
	Mean \pm SE (mg)	Mean \pm SE (mg)
Human TF	60.69 \pm 12.30	28.93 \pm 3.00
Exogenous serotonin (6 μM)	21.98 \pm 3.50	17.07 \pm 0.89
Human TF after p-Chlorophenylalanine	17.64 \pm 2.63	14.06 \pm 1.72
Human TF after methysergide	14.4 \pm 2.1	12.4 \pm 3.2

These data suggest that the lymph-node increasing effect of TF may be dependent on the local release of endogenous serotonin by tissue mast cells or nerve endings, and not to the direct effect of exogenous serotonin contained in TF as was originally surmised. Since vasoactive amines are essential for the production of delayed type hypersensitivity,⁶ it is probable that TF effects the hemodynamics of the lymph node microvasculature in such a way as to favor increased cell migration and interaction.

The dose response of the lymph node to TF, injected in 0.1-ml aliquots, peaks at a 10-fold dilution of crude TF. Node-weight increasing effects are minimal but, still present at a 10,000-fold dilution; 1X and 10X concentrations do not cause further increases.

PICLC + KVEE. Table XII depicts lymph node enlargement following exposure to KVEE with and without PICLC. KVEE alone caused a gradual increase in node weight which peaked around the 7th day after injection. When KVEE was given in combination with PICLC a more rapid rise and prolonged increase in node weight occurred.

TABLE XII. SEQUENTIAL CHANGES IN LYMPH NODE WEIGHT FOLLOWING ANTIGEN/ADJUVANT INJECTIONS

DAYS AFTER INJECTION	NODE WEIGHT (mg)		
	KVEE DRAINING	KVEE + PICLC DRAINING	CONTROL
0	18	19	18
1	21	38	19
2	25	56	17
3	28	48	18
7	43	45	20
14	38	43	18
28	28	44	17
35	21	38	19

Lymphocyte traffic was determined in the same nodes using the LMI (Table XIII). The influx of lymphocytes induced KVEE alone was short-lived and moderate in magnitude while increased traffic was prolonged with adjuvant or antigen-adjuvant combinations.

TABLE XIII. LYMPHOCYTE TRAFFIC INDUCED BY KVEE WITH AND WITHOUT ADJUVANT

DAYS	LMI			CONTROL
	KVEE	KVEE + PICLC	PICLC	
0	0.80	0.90	1.00	0.89
1	1.18	1.49	1.51	0.75
2	1.21	1.80	1.48	0.76
3	1.00	1.48	1.49	0.80
7	0.90	1.46	1.43	0.81
14	0.88	1.48	1.20	0.69
28	0.89	1.25	1.10	0.73
35	0.87	1.10	1.00	0.75

In another group of rats (age, weight and sex matched litter-mates) viral plaque inhibition titers were measured sequentially for 35 days after immunization with KVEE and KVEE-PICLC combinations. The data clearly show that plaque inhibition titers after immunization with KVEE + PICLC were nearly 20 times those seen for KVEE alone (Table XIV). No significant

advantage was seen when KVEE was given in one side and PICLC was given in the contralateral side.

TABLE XIV. VIRAL PLAQUE INHIBITION TITERS FOLLOWING VACCINATION WITH KVEE ALONE AND IN COMBINATION WITH PICLC

VACCINE	RECIPROCAL TITER BY DAYS					
	3	7	10	14	24	35
KVEE	13	107	430	22	107	1217
KVEE + PICLC	32	2048	4096	1217	1448	16384
KVEE (ipsilateral) + PICLC (contralateral)	16	608	304	86	304	1217

TF + KVEE. A new group of animals were used for these studies and some data about the comparative effectiveness of PICLC and TF were needed; therefore, the previous studies were repeated as additional controls for the TF experiments. Since morphological studies of the lymph node effects of human TF demonstrated marked accumulation of small lymphocytes in the paracortex and "activation" of sinusoidal macrophages, it seemed likely that TF would be useful as an adjuvant when used with KVEE. To test this hypothesis 0.1 ml of a suspension of TC-83 KVEE (Lot E96) was injected SC into the lateral thorax. Sequential measurement of KVEE-induced node weight changes resulted in a modest trapping response at 24-48 hr followed by a gradual rise to peak weight at 7 days postinjection. Sequential plaque neutralization (PN) titers for VEE were also modest and short-lived. The TF which was injected (with KVEE) in the first experiment was from a donor who had not been exposed to VEE vaccine (Table XV).

TABLE XV. VIRAL PLAQUE INHIBITION TITERS FOLLOWING VACCINATION WITH KVEE ALONE AND IN COMBINATION WITH TF FROM TWO SOURCES

DAY	RECIPROCAL TITER				
	EXPERIMENT 1		EXPERIMENT 2		
	KVEE CONTROL	KVEE + TF (VEE-)	KVEE CONTROL	KVEE + TF (VEE+)	KVEE + PICLC
3	16	6	11	10	8
7	-	-	13	23	40
10	20	5	10	16	32
14	8	8	8	11	16
24	5	7	32	128	362
35	6	8	64	1024	4096

Although these lymph nodes rapidly enlarged at 24-48 hr and remained so for 14 days, the PN titers were less than or equal to the titers for KVEE alone. The second time this experiment was performed the TF donor had been previously exposed to TC-83 VEE vaccine. Rapid lymph node enlargement occurred, and the PN titers for the KVEE + TF recipient rats were 2-3 times the KVEE controls. PICLC was used in combination with KVEE and produced comparable titer increases.

Similar experiments in splenectomized rats showed that PICLC achieved immune potentiation locally in the regional node while TF probably worked locally and systemically. Further studies of the influence of PICLC and TF on the immune response to KVEE are in progress. In addition, other biological factors such as tuftsin, thymosin, and prostaglandin will be tested for adjuvanticity.

Presentations:

1. Anderson, A. O. Lymphocytes: recirculation, lymph node structure, T and B cells, and cell cooperation. Presented, The Johns Hopkins Immunology Council Survey Course on the Pathogenesis of Allergic and Infectious Diseases, Lecture #10, Johns Hopkins University, Baltimore, MD, 24 Feb 1976.
2. Anderson, A. O. and N. D. Anderson. The mechanism of lymphocyte homing. Presented, The American Association of Immunologists, FASEB Symposium, Anaheim, CA, 12 Apr 1976 (Fed. Proc. 35:351, 1976).

Publications:

1. Anderson, A. O., N. D. Anderson, and R. G. Wyllie. 1975. Studies on the structure and permeability of the microvasculature in normal rat lymph nodes. *Am. J. Pathol.* 80:387-418.
2. Anderson, N. D., A. O. Anderson, and R. G. Wyllie. 1975. Microvascular changes in lymph nodes draining skin allografts. *Am. J. Pathol.* 81:131-160.
3. Anderson, A. O., and N. D. Anderson. 1976. Lymphocyte emigration from high endothelial venules in rat lymph nodes. *Immunology* 31: in press.
4. Anderson, N. D., A. O. Anderson, and R. G. Wyllie. 1976. Specialized structure and metabolic activities of high endothelial venules in rat lymphatic tissues. *Immunology* 31: in press.

LITERATURE CITED

1. Gowans, J. L. 1959. The recirculation of lymphocytes from blood to lymph in the rat. *J. Physiol.* 146:54-69.
2. Anderson, N. D., A. O. Anderson, and R. G. Wyllie. 1975. Microvascular changes in lymph nodes draining skin allografts. *Am. J. Pathol.* 81:131-160.
3. Anderson, A. O., N. D. Anderson, and R. G. Wyllie. 1974. Lymphocyte emigration from high endothelial venules (HEV). *Fed. Proc.* 33:628.
4. Anderson, A. O., N. D. Anderson, and R. G. Wyllie. 1975. Studies on the structure and permeability of the microvasculature in normal rat lymph nodes. *Am. J. Pathol.* 80:387-418.
5. Visa, D., J. M. Goust, R. Moulias, L. K. Trejdosiewicz, A. Collard, and N. Müller-Berat. 1975. In vitro production of transfer factor by lymphoblastoid cell lines. *Transplant. Proc.* 7 (Suppl. 1):329-333.
6. Gershon, R. K., P. W. Askenase, and M. D. Gershon. 1975. Requirement for vasoactive amines for production of delayed-type hypersensitivity skin reactions. *J. Exp. Med.* 142:732-747.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ⁹	2. DATE OF SUMMARY ¹⁰	REPORT CONTROL SYMBOL	
3. DATE PREV SURVEY	4. KIND OF SUMMARY	5. SUMMARY ACTV ¹¹	6. WORK SECURITY ¹²	DA OF6426	76 07 01	DD-DR&E(AR)636	
76 01 30	D. CHANGE	U	U	NA	NL	7. REGRADING ¹³	
8. NO./CODES ¹⁴	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	8. SPECIFIC DATA - CONTRACTOR ACCESS			
9. PRIMARY	61101A	3A161101A91C	00	<input checked="" type="checkbox"/> YES	<input type="checkbox"/> NO	9. LEVEL OF SUM	
10. CONTRIBUTING				A. WORK UNIT			
c. 114(e)(f)	CARDS 114(e)(f)			135 834/02/014			
11. TITLE (Punctuate with Security Classification Code) (U) Evaluation of Vitafiber (R) system for use in continuous cell culture							
12. SCIENTIFIC AND TECHNOLOGICAL AREA ¹⁵ 003500 Clinical medicine; 004900 Defense; 010100 Microbiology							
13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD				
76 01	CONT	DA	C. In-house				
17. CONTRACT/GRAANT							
18. DATES/EFFECTIVE:	EXPIRATION:	19. RESOURCES ESTIMATE	20. PROFESSIONAL MAN YRS				
19. NUMBER ¹⁶ :	NA	FISCAL YEAR	76	21. FUNDS (in thousands)			
20. TYPE:	4. AMOUNT:	CURRENT	0.1	10.0			
21. KIND OF AWARD:	5. CUM. AMT.	77	0.5	20.0			
22. RESPONSIBLE DOG ORGANIZATION							23. PERFORMING ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701							NAME: Pathology Division USAMRIID ADDRESS: Fort Detrick, MD 21701
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. RANK: . TELEPHONE: 301 663-2833							PRINCIPAL INVESTIGATOR (Punctuate each name with Security Classification Code) NAME: Johnson, A. D. TELEPHONE: 301 663-7211 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME:
24. GENERAL USE Foreign intelligence considered							POC:DA
25. REFERENCES (Punctuate each name with Security Classification Code) (U) BW defense; (U) Tissue culture; (U) Vaccines; (U) Viruses; (U) Rickettsiae; (U) Military medicine							26. TECHNICAL OBJECTIVE ¹⁷ , 26. APPROACH, 26. PROGRESS (Punctuate individual paragraphs identified by number. Punctuate each name with Security Classification Code.)
23. (U) Assess practicality of the Vitafiber system for growth of viral material to be used in vaccine production. New vaccines against many organisms, especially those of potential BW importance, are needed by the military forces. The Vitafiber system is being evaluated to determine reduction in space, time, and cost involved for vaccines with sufficiently high titer. Standard methods of vaccine production are serving as controls for evaluation of the method.							24. (U) After it has been shown that chick fibroblasts will grow in this commercial system, determine optimal conditions for cell production and vaccine virus growth on the cell line.
25. (U) 76 01 - 76 06 - A small artificial unit, composed of a bundle of 150 fibers, has been produced by Amicon under the trade name, Vitafiber. Medium is perfused through the fibers, equilibrates through the pores, and nourishes the cells growing on the outside of the fibers.							Comparative studies were conducted using the Vitafiber system and standard roller bottle. Virus titers ranged from 5 to 20 million PFU/ml. Total yield of virus from one Vitafiber approximately equals that from one roller bottle. Studies from units grown for longer periods of time (up to 7 days) indicate that the Vitafiber systems may be capable of continued production of virus, whereas roller bottle cultures are routinely destroyed after 24 hr. Virus harvested from the Vitafiber appears to be free of extracellular debris, while that from roller bottle cultures is harvested with much cell debris, necessitating further purification.

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PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

* U.S. GPO: 1974-840-843/0091

BODY OF REPORT

**Project No. 3A061101A91C: In-House Laboratory Independent Research (U)
(3A762760A834):**

**Task No. 3A061101A91C 00:
(3A762760A834 01): (Prevention and Treatment of Biological Agent
Casualties)**

**Work Unit No. 910 00 135: Evaluation of Vitafiber^R System for Use in
(834 02 014): Continuous Cell Culture**

Background:

Production of virus in high titer has been accomplished by growth in roller bottles. Monolayer cultures of suitable cell lines, such as chick fibroblasts, BHK-21 (baby hamster kidney), or other continuous cell lines, are infected with virus, grown for about 24 hr, and virus recovered. With the group A arbovirus (as typified by VEE), the virus is released from the cell and can be recovered from the supernatant. Cell destruction by the virus is complete, and the debris accumulated from the cytopathic effect complicates preparation of clean virus.

An artificial membrane surface, suitable for cell growth, has been manufactured by Amicon. The Vitafiber^R units have 150 fibers in a closed-end bundle. Vitafiber is plastic, autoclavable, and about 15 cm long. Medium can be perfused through the fiber bundle to nourish the cells which attach and grow on the outer surface of the fibers. Cellular wastes are removed by the same method. The molecular weight cut-off of the fibers is 100,000, allowing diffusion of medium constituents (except globulins present in serum) across the cell layer.

Preliminary work with artificial capillary systems for cell growth has shown that the production of cells and/or by-products may be enhanced by this method. Knazek et al.¹ reported that increased yields of human chorionic gonadotropin were related to increased numbers of cells present in the system, as well as to the in vivo-like environment provided by the artificial capillary system. Continuous nutrient supply and waste product removal provide a better environment for the cells being cultured. Contact inhibition seems to be abolished; some 3-dimensional effects have been obtained with mouse fibroblasts.²

Progress:

Chick fibroblasts have been grown successfully in the Vitafiber artificial unit for periods of 3-10 days. BHK-21 cells have been grown in the Vitafiber for a 7-day period.

The ability of the cells to produce virus was chosen as a suitable assay for cell viability. VEE vaccine strain TC-83 was utilized in the Vitafiber system. Samples of supernatant were assayed for virus by plaquing technique.³

In the first set of experiments, chick fibroblast slurry was seeded onto the fibers. The reservoir was filled with medium E199, with 10% fetal calf serum added. The extracapillary space of the Vitafiber units contain 2.6 ml. Originally 1-ml samples were removed periodically for virus titration. Recovery of virus was found to be directly related to the number of cells seeded into the unit and to the virus titration of the inoculum (Table I).

TABLE I. RECOVERY OF VEE FROM CHICK FIBROBLASTS GROWN IN VITAFIBER AT 24 HR

CHICK FIBROBLAST SEED SLURRY (cells)	INOCULUM $\text{LOG}_{10}/\text{ml}$ TC-83 VACCINE	YIELD ^a ($\text{Log}_{10}/\text{ml}$)
1.6×10^8	5.5	9.7
1.7×10^8	6.5	10.9
3.7×10^7	6.5	9.6
1.7×10^7	6.5	9.7
3.7×10^6	6.5	8.6
2.3×10^8	5.9	9.9
1.1×10^9	5.9	10.1

^a Sample size 1.0 ml.

In order to obtain a more accurate picture of the total amount of virus produced, 10-ml aliquots of medium were used to flush through the extra-capillary space of the Vitafiber. Sampling was begun at 22 hr and continued hourly for 8 hr. Additional samples were collected at 48 and 72 hr. About 2.1×10^{12} PFU of virus were collected by this procedure (Table II).

TABLE II. RECOVERY OF VEE FROM CHICK FIBROBLASTS BY PERIODIC SAMPLING

SAMPLE TIME (hr)	YIELD ^a (\log_{10}/ml)
0 (Inoculum)	5.9
22	10.0
23	9.5
24	9.3
25	9.5
26	8.1
27	8.3
28	8.0
29	8.9
48	9.1
72	8.0

^a Sample size 10 ml.

Two Vitafiber systems were set up with chick fibroblasts to be sacrificed for histologic examination. One unit was infected with TC-83 and grown for 22 hr. This unit, and the control unit were infiltrated with warm agarose to preserve the integrity of the fiber bundle. The bundle was cut and sections were prepared for fluorescence (frozen sections), for routine histologic examination and for electron microscopy.

Light microscopy showed growth of the chick fibroblasts between the fibers of the bundle. Some cells were growing in the convoluted surface of the fibers. The fibroblasts did not completely fill the spaces between the fibers, indicating that more cells could be accommodated by the Vitafiber unit. The virus-infected unit showed necrotic areas, typical of VEE.

Publications:

None.

LITERATURE CITED

1. Knazek, R. A., P. O. Kohler, and P. M. Gullino. 1974. Hormone production by cells grown in vitro on artificial capillaries. *Exp. Cell Res.* 84:251-254.
2. Knazek, R. A., P. M. Gullino, P. O. Kohler, and R. L. Dedrick. 1972. Cell culture on artificial capillaries: an approach to tissue growth in vitro. *Science* 178:65-67.
3. U.S. Army Medical Research Institute of Infectious Diseases. 1 July 1975. Annual Progress Report, FY 1975. p. 449-453. Fort Detrick, Md.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ⁹ DA 0D6425	2. DATE OF SUMMARY ⁹ 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SURVEY 75 08 18	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY ⁹ U	6. WORK SECURITY ⁹ U	7. REGADING ⁹ NA	8. DISC'DN INSTR'N NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: a. PRIMARY b. CONTRIBUTING c. Contributing	PROGRAM ELEMENT 62760A	PROJECT NUMBER 3A762760A834		TASK AREA NUMBER 02	WORK UNIT NUMBER 103	
11. TITLE (Pursue with Security Classification Code) (U) Host resistance to facultative bacteria						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁹ 003500 Clinical medicine; 004900 Defense; 010100 Microbiology						
13. START DATE 72 11	14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house		
17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS		20. FUNDS (in thousands)
21. DATES/EFFECTIVE:		EXPIRATION:		FISCAL YEAR	PREVIOUS CURRENT	321.3
22. NUMBER: NA		23. AMOUNT: F. CUM. AMT.		77	3.0	245.0
24. RESPONSIBLE DOD ORGANIZATION		25. PERFORMING ORGANIZATION				
NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		NAME: Bacteriology Division USAMRIID ADDRESS: Fort Detrick, MD 21701				
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Puruse with Security Classification Code) NAME: Hunter, D. H. TELEPHONE: 301 663-7341 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: Janssen, W. A. NAME: McGann, V. G.		POC:DA		
26. KEYWORDS (Pursue EACH with Security Classification Code) (U) BW defense; (U) Military medicine; (U) Cell-mediated immunity; (U) Francisella tularensis; (U) Salmonella typhimurium; (U) Syngeneic mice						
27. TECHNICAL OBJECTIVE, ⁹ 28. APPROACH, 29. PROGRESS (Puruse individual paragraphs identified by number. Pursue text of each with Security Classification Code.)						
23 (U) Evaluate potential for enhancement of nonspecific and/or specific host resistance and define the role of humoral and cell-mediated immunity in facultative intracellular infections. This will lead to important information regarding treatment of and prophylaxis against BW agents.						
24 (U) Quantitate differences in specific and nonspecific antibacterial resistance in mice and explore feasibility of regulating immune response. Using passive transfer of immune serum and/or lymphoid cells to syngeneic recipients with fully competent or selectively depressed immune mechanisms, determine resistance attained by active and passive immunization of <i>F. tularensis</i> challenge of graded virulence.						
25 (U) 75 07 - 76 06 - Passively transferred spleen cells from AKR/J mice immunized with live tularemia vaccine ensure high level protection (nearly 100% survival) to nonimmune recipients against challenge with fully virulent <i>F. tularensis</i> . The protection is antigen-dependent. Specific protection against IP and IC challenge can be enhanced and recalled for extended time periods with appropriate concentration of homologous killed antigen. Short-term nonspecific protection against IP but not SC challenge can be elicited by appropriate concentrations of heterologous killed antigen. This model system permits definitive studies on mechanisms involved in effective control of infections caused by highly virulent facultative intracellular bacteria. Nonspecific host resistance to <i>L. monocytogenes</i> and <i>S. typhimurium</i> induced in the AKR/J mouse by live tularemia and DPT vaccines suggests that effective host resistance to infectious diseases of military significance may be induced rapidly and effectively by vaccines currently in medical use.						
Publication: Infect. Immunity 12:999-1005, 1975.						
Available to contractors upon contractor's approval.						

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 103: Host Resistance to Facultative Bacteria

Background:

During the past decade, significant contributions have been made toward defining the role of cell-mediated (CMI) and humoral immunity in resistance to infections caused by facultative intracellular bacteria. Cellular factors have been allocated the primary role to the virtual exclusion of the humoral system. The evidence is based primarily on reduction in growth of the infecting microorganism in the spleen and liver or by a delay in time to death of laboratory animals which, at the time of challenge, had received dissociated spleen cells and/or macrophages from immune donors (whose serum was found to be inert in passive protection tests). In our previously described studies^{1,2} we demonstrated that under appropriate conditions, passively transferred spleen cells from immunized mice will provide high-grade protection (survival approaching 100%) to nonimmune recipients against IV, IP or SC challenge with a fully virulent strain of Francisella tularensis.

Facultative intracellular bacteria have been reported to elicit protection against the specific etiologic agent and also against antigenically unrelated microorganisms.^{3,4} This nonspecific protection is dependent upon increased microbicidal activity of mononuclear phagocytes. Acquisition of data on the spectrum, degree, duration, recall or enhancement of nonspecific resistance as well as comparative information on specific resistance in an appropriate animal model will provide a firm basis for critical assessment of the potential for providing a practical, rapid and effective means for development of non-specific resistance to several infectious diseases of military significance.

Progress:

I. Our initial goal, to provide a model that would afford syngeneic recipients of immune spleen cells (ISC) from immunized donors survival against infection with a fully virulent strain of F. tularensis, has been accomplished.^{1,2} The experimental model consists of: (1) primary immunization of 11-14-wk-old AKR/J mice with live tularemia vaccine; (2) secondary immunization on day 30 with virulent strain SCHU S4; (3) 12 days after booster, immune spleen cell transfer to syngeneic recipients that 20 hr later were challenged by the IP, SC, or IV route with 25 to 50 MLD of streptomycin-resistant strain SCHU S5; and (4) administration of streptomycin therapy for 5 days from time

of spleen cell transfer. Evidence was also presented that the protection was adoptive and that specific antigen was required for maximal expression of immunity. Efforts during the current year were directed toward determining the duration of adoptive immunity and identifying the quantity and specificity of antigen required for transfer of immunocompetence.

Duration of the capability to transfer adoptive immunity in the murine model. Female AKR/J mice were vaccinated SC with 10^3 *F. tularensis* strain LVS and boosterized 30 days later with 10^3 strain SCHU S4 by the IP route. Immune spleen cells were prepared as previously described² and transferred to syngeneic recipients on selected days following the booster (Table I).

TABLE I. DURATION OF ABILITY OF AKR/J MOUSE SPLEEN CELLS FROM LVS VACCINATED SCHU S4 BOOSTERIZED VACCINEES^a TO TRANSFER IMMUNITY TO SYNGENEIC RECIPIENTS.

DONOR CELLS TRANSFERRED				RECIPIENT SURVIVAL ^b S/T
Postbooster (Day)	Spleen cells ($\times 10^7$)	Spleen cell-associated live <i>F. tularensis</i> SCHU S4		
4	20	< 10^4		10/10
6	6	10^6		0/10
8	10	10^2		10/10
10	14	50		8/10
12	7	16		9/10
14	11	86		7/10
18	10	0		10/10
21	8	1		6/10
35	7	0		4/10
45	6	0		1/15
47	7	0		0/7
47	$7 + 10^7$ Ag ^c	0		7/8

^a Vaccinated with 10^3 LVS SC; boosterized in 30 days with 10^3 SCHU S4 IP.

^b AKR/J recipients challenged IP with 25-30 MLD of streptomycin-resistant SCHU S5 and administered 400 µg streptomycin twice daily for 5 days (survivors/total). All challenge controls died within 6 days.

^c 10^7 dialyzed phenol-killed SCHU S4 in 0.2 ml RPMI 1640 administered IP at time of spleen cell transfer.

ISC recipients were challenged 20 hr after transfer with 25-30 MLD of streptomycin-resistant *F. tularensis* SCHU S5 and administered 400 µg streptomycin twice daily for 5 days. The results presented in Table I indicate that no protection was provided by 6-day postbooster ISC in contrast to the solid protection afforded by ISC transferred on days 4 and 8. It is postulated that the spleen cell-associated SCHU S4 present in the 6-day donor cells (10^6 viable organisms) constituted so formidable a burden to the recipients that, despite streptomycin therapy, the protective potential of the immune spleen cells was masked. Immune spleen cells transferred 21, 35, 45 or 47 days postbooster were virtually devoid of live cell-associated SCHU S4 and their recipients evidenced decreased protection, i.e., 60, 40, 7 and 0% survival respectively. In the transfer performed at 47 days postbooster 1 group of 8 recipients, in addition to receiving ISC, was concurrently injected by the IP route with 10^7 dialyzed phenol-killed SCHU S4 in 0.2 ml RPMI 1640. The 47-day ISC alone provided no protection; however, in the presence of specific but nonviable antigen, nearly complete protection was afforded the syngeneic recipients. These data confirm the capability of ISC to transfer adoptive immunity under the conditions described persists for at least 18 days and that recall of this capability can be effected as late as 47 days by stimulation with specific nonviable antigen.

Protection afforded syngeneic recipients of ISC from LVS vaccinated-LVS boosted AKR/J mice. In the original studies to establish the murine model for evaluation of CMI in tularemia,^{1,2} the LVS vaccinated-LVS boosted donor ISC did not provide protection to syngeneic recipients at 3, 6, 12 or 19 days following a LVS booster. Also noted was the absence of any viable cell-associated bacteria in the ISC preparations. Since we subsequently demonstrated that antigen, either live cell-associated or killed bacterial cells, is required for ISC to express their protective activity, reexamination of this potentially superior model appeared warranted.

Donor AKR/J mice were vaccinated SC with 10^3 LVS and boosted 27 days later with 10^6 LVS IP. Immune spleen cell transfers to 2 groups of syngeneic recipients were performed at 4, 6, 9, 10 and 29 days following booster; one of the groups was also inoculated IP with killed SCHU S4 cells at time of transfer. All recipients were challenged IP 20 hr later with 25-135 MLD streptomycin-resistant *F. tularensis* SCHU S5 and administered 400 µg streptomycin twice daily for 5 days. In groups that received ISC alone, only those recipients of ISC with cell-associated live LVS (4 and 6 days) were protected against challenge with SCHU S5 (Table II). In contrast, when 10^7 killed SCHU S4 antigen was injected concurrently, survival in all groups approached 100%. These data sustain our postulate concerning the requirement for continued presence of antigen for expression of adoptive immunity; in addition, they strongly suggest that the LVS vaccinated-LVS boosted murine model offers great potential for study of the mechanisms involved in cell-mediated immunity since protection is now possible with ISC preparations free of viable organisms.

TABLE II. TRANSFER OF IMMUNITY TO AKR/J RECIPIENTS OF SYNGENEIC SPLEEN CELLS FROM LVS VACCINATED-LVS BOOSTERED DONORS:^a INFLUENCE OF KILLED ANTIGEN ON IMMUNE COMPETENCE OF SPLEEN CELLS.

Postbooster (Day)	DONOR CELLS TRANSFERRED		RECIPIENT SURVIVAL ^b	
	Spleen cells ($\times 10^7$)	Spleen-cell- associated live <i>F. tularensis</i>	No antigen added	Antigen added ^c
4	9	9	8/10 ^d	10/10
6	7	1	2/10	9/10
9	8	0	0/10	10/10
10	6	0	0/10	9/10
29	6	0	1/8	7/8

^a Donor AKR/J mice vaccinated SC with 10^3 LVS; boosted in 27 days with 10^6 LVS IP.

^b AKR/J recipients challenged IP with 25-135 MLD streptomycin-resistant SCHU S5; all controls died within 10 days after challenge. Recipients administered 400 μ g streptomycin twice daily for 5 days.

^c 10^7 dialyzed phenol-killed SCHU S4 in 0.2 ml RPMI 1640 administered IP at time of spleen cell transfer.

^d Survivors/total.

Quantity of antigen required for maximal expression of adoptive immunity. Donor AKR/J mice were immunized with 10^3 viable LVS SC and boosted 33 days later with 10^6 viable LVS IP. Spleen cell transfer was made on day 9 post-booster. Each syngeneic recipient received 9.6×10^7 spleen cells IP, followed by IP injection with 0.2 ml of saline or of 10^2 - 10^9 dialyzed phenol-killed SCHU S4. Since previous experiments with the LVS-LVS immunized donor system indicated that viable organisms were not detectable in a spleen cell suspension by 7 days following booster, recipients were not given streptomycin therapy. One group of recipients was challenged IP 20 hr later with 27 organisms of virulent strain SCHU S5 and another comparable group was challenged SC on day 30 post transfer with 114 SCHU S4 organisms. Control groups included dose controls and recipients of normal spleen cells plus antigen (antigen dose: 10^7 , 10^8 and 10^9). All control animals died within 7 days and there were no significant differences in time of death between groups. Results presented in Table III show that: (1) antigen was

required for maximal expression of adoptive immunity; (2) maximal antigen effect was achieved with 10^6 - 10^8 bacteria; (3) protection afforded recipients by immune spleen cells alone was adoptive, i.e., survivors succumbed to backchallenge; and (4) active immunity developed in recipients that were administered antigen doses $> 10^6$ killed bacteria at the time of ISC transfer. Data from control groups indicated that killed antigen alone or in combination with normal spleen cells does not protect against virulent challenge either initially or at 30 days.

TABLE III. ANTIGENIC REQUIREMENT FOR MAXIMAL EXPRESSION OF ADOPTIVE IMMUNITY IN AKR/J RECIPIENTS OF SYNGENEIC SPLEEN CELLS FROM LVS VACCINATED DONORS ADMINISTERED A LVS BOOSTER.^a

ANTIGEN DOSE (Killed SCHU S4)	20 HR CHALLENGE GROUP		30-DAY CHALLENGE GROUP ^c
	20 hr challenge ^b	30 day backchallenge ^c	
None	5/10 ^d	0/5	0/10
10^2	7/10	0/7	0/10
10^4	9/10	1/9	3/10
10^6	9/10	8/9	6/10
10^7	10/10	10/10	4/10
10^8	10/10	10/10	9/10
10^9	2/10	2/2	8/10

^a Vaccinated with 10^3 LVS SC; boosted 33 days later with 10^6 LVS IP; 1×10^8 spleen cells transferred 9 days postbooster.

^b Recipients challenged IP with 27 MLD of F. tularensis SCHU S5. All control mice died within 7 days.

^c SC challenge with 114 MLD of F. tularensis SCHU S4. All challenge groups died within 6 days.

^d Survivors/total.

These results establish that a finite quantity of antigen is required for maximal expression of CMI and confirm previous observations that survivors from experimental groups administered immune spleen cells and 10^7 killed SCHU S4 bacteria were resistant to backchallenge. Induction of active immunity by administration of immune spleen cells plus antigen cannot be explained at the present time; however, in vivo production of transfer factor or lymphokines may be involved. Survival of 5 of 10 mice in the group that received spleen cells without antigen was unexpected; however, review of our previous experience indicated that death patterns for IP challenged groups were characteristically more variable than those for groups challenged by either the IV or SC route. These impressions were confirmed when the antigen titration was repeated with comparable groups of immune spleen cell recipients challenged IP and SC (Table IV).

TABLE IV. INFLUENCE OF THE ROUTE OF CHALLENGE ON DEMONSTRATING THE ANTIGENIC REQUIREMENT FOR MAXIMAL EXPRESSION OF ADOPTIVE IMMUNITY IN AKR/J RECIPIENTS OF SYNGENEIC SPLEEN CELLS FROM LVS VACCINATED DONORS ADMINISTERED A LVS BOOSTER.^a

ANTIGEN DOSE (Killed SCHU S4)	CHALLENGE ROUTE			
	IP		SC	
	20-hr challenge ^b	30-day backchallenge ^c	20-hr challenge	30-day backchallenge
None	3/10 ^d	0/3	0/10	--
10 ²	--	--	0/10	--
10 ⁴	1/10	0/1	2/10	2/2
10 ⁶	7/10	6/7	7/10	7/7
10 ⁷	8/10	8/8	9/10	9/9
10 ⁸	4/10	3/4	8/10	8/8
10 ⁹	3/10	3/3	6/10	6/6

^a Vaccinated with 10³ LVS SC: boosted 29 days later with 10⁶ LVS IP; 10⁸ spleen cells transferred 9 days postbooster.

^b Recipients challenged IP or SC with 25 MLD of F. tularensis SCHU S5.

^c SC challenge with 150 MLD of F. tularensis SCHU S4.

^d Survivors/total.

Specificity of the antigenic requirement for expression of maximal adoptive immunity in AKR/J recipients of syngeneic spleen cells from LVS-vaccinated donors. In order to examine the immunologic specificity of the antigenic requirement for continued expression of adoptive immunity by LVS-LVS immune spleen cells, the following experiment comparing killed antigens from F. tularensis SCHU S4 and Yersinia pestis was conducted. Donor AKR/J mice were immunized with 10³ viable LVS SC and boosted 1 mon later with 10⁶ viable LVS IP. Spleen cell transfer was made 8 days postbooster. Each recipient received ~10⁸ spleen cells IP concurrently with IP injection of killed antigen (10⁶ - 10⁸ whole cells) contained in 0.2 ml of saline. The antigens used were phenol-killed F. tularensis SCHU S4, and Y. pestis from the standard Cutter vaccine. Each antigen was washed 3 X in sterile physiologic saline solution (PSS) and brought to appropriate concentrations in PSS. Recipients were challenged with F. tularensis SCHU S5, by the IP or SC route 1 and 7 days following ISC transfer; all survivors were back-challenged SC approximately 1 mon later with 10² F. tularensis SCHU S4.

Control groups included challenge-dose controls, recipients of normal spleen cells with and without added antigen, and antigen alone. All control animals died within 7 days; there were no significant differences in time to death between groups.

The results presented in Table V show that, when recipient mice were challenged by the IP route 20 hr following immune spleen cell transfer, *Y. pestis* antigen was as effective as *F. tularensis* antigen in potentiating protective activity of the ISC. This protective effect of *Y. pestis* antigen was no longer evident when survivors were backchallenged, nor when primary challenge was delayed for 1 mon (not shown in Table).

TABLE V. SPECIFICITY OF THE ANTIGENIC REQUIREMENT FOR EXPRESSION OF ADOPTIVE IMMUNITY IN AKR/J RECIPIENTS OF SYNGENEIC SPLEEN CELLS FROM LVS-VACCINATED DONORS.^a

KILLED ANTIGEN ^b	DOSE	CHALLENGE GROUP			
		1 Day		7 Day	
		Initial challenge ^c	30-day backchallenge ^d	Initial challenge	30-day backchallenge ^d
<u><i>F. tularensis</i></u>	10^6	7/10 ^e	6/7	--	
	10^7	8/10	8/8	10/10	10/10
	10^8	4/10	3/4	--	
<u><i>Y. pestis</i></u> Cutter vaccine	10^6	4/10	1/4	1/10	0/1
	10^7	8/10	0/8	6/10	0/6
	10^8	3/10	1/3	9/10	0/9
None	--	3/10	0/3	1/10	1/1

^a Donors vaccinated with 10^3 LVS SC and boosted 29 days later with 10^6 LVS IP; 10^8 spleen cells transferred 8 days postbooster.

^b *Y. pestis* antigen: washed Cutter vaccine; *F. tularensis* vaccine: phenol-killed and washed *F. tularensis* SCHU S4. The antigens contained in 0.2 ml of saline were administered IP to recipients at time of cell transfer.

^c Recipients challenged IP with 25 MLD of *F. tularensis* SCHU S5. All controls died within 7 days.

^d Recipients backchallenged SC with 130 - 150 MLD of *F. tularensis*, SCHU S5. All challenged controls died within 6 days.

^e Survivors/total.

The mechanism of protective activity potentiated by killed plague bacilli is not readily explained. Two possibilities are: (1) some major antigenic component shared by both *Y. pestis* and *F. tularensis*, or (2) nonspecific activity caused by the close proximity of killed antigen, ISC and challenge organisms within the peritoneal cavity. The observation that surviving recipients of ISC and *Y. pestis* antigen were not protected when back-challenged or when challenge was delayed for 31 days tends to mitigate against the presence of a major antigenic cross reaction between the 2 organisms. The possibility remains that plague organisms initiate high level activation of macrophages within the peritoneal cavity, and close proximity of ISC with such highly activated macrophages may be required for protection against SCHU challenge.

Results indicating that an increased amount of plague antigen was necessary to obtain protection when primary challenge was delayed 7 days tends to support this hypothesis.

When recipient mice were challenged by the SC route at selected days following ISC and antigen transfer, no protective potentiation by *Y. pestis* antigen was evident (Table VI).

TABLE VI. SPECIFICITY OF ANTIGENIC REQUIREMENT FOR ADOPTIVE TRANSFER OF IMMUNITY IN AKR/J RECIPIENTS CHALLENGED SC AT VARIOUS INTERVALS FOLLOWING ISC TRANSFER.^a

SC CHALLENGE WITH 25 MLD SCHU S5 (day after cell transfer) ^b	RECIPIENT TREATMENT WITH KILLED ANTIGEN (10^7 organisms) ^c			<i>F. tularensis</i> -treated recipients	
	Untreated recipients	<i>Y. pestis</i> -treated recipients	Initial challenge	30-day backchallenge ^d	
1	0/10 ^e	0/10	6/10	6/6	
3	0/10	0/10	8/10	8/8	
5	0/10	0/10	10/10	10/10	
10	0/10	0/10	10/10	9/10	
14	0/10	0/10	9/10	9/9	
29	0/2	0/2	3/4	ND	

^a Donors vaccinated with 10^3 LVS SC and boosted 29 days later with 10^6 LVS IP; 10^8 spleen cells transferred 8 days postbooster.

^b All controls died within 8 days.

^c *Y. pestis* antigen: washed Cutter vaccine; *F. tularensis* vaccine: phenol-killed and washed *F. tularensis* SCHU S4. The antigens contained in 0.2 ml of saline were administered IP to recipients at time of cell transfer.

^d SC challenge with 127 MLD *F. tularensis*, SCHU S4.

^e Survivors/total.

These results indicate that the requirement for antigenic stimulation of ISC is immunologically specific and that the protective activity induced by nonspecific antigens (Y. pestis) in IP challenged recipients permits recognition of the contribution of nonspecific elements involved in host defense mechanisms.

The potentiating effect of 2 other antigenically unrelated organisms was examined in similar fashion. The antigens used were Salmonella typhi from the standard typhoid vaccine and Bordetella pertussis from the standard DPT vaccine. Each antigen was washed 3 X and brought to appropriate concentrations in PSS. Recipients were challenged IP with F. tularensis SCHU S5, at 1 and 7 days following ISC transfer; all survivors were backchallenged SC approximately 1 mon later with 10^2 F. tularensis SCHU S4. The results, presented in Table VII, indicate that when recipient mice are challenged IP 20 hr and 7 days following ISC transfer, B. pertussis and S. typhi antigens were as effective as Y. pestis antigen in potentiating the protective activity of ISC. As observed with Y. pestis antigen, this protective effect was no longer evident when survivors were backchallenged. Two experiments currently in progress compare IP and SC challenge routes and the efficacy of killed S. typhi and B. pertussis antigens. Preliminary results indicate that the nonspecific protective effect evident upon IP challenge is no longer apparent with SC challenge.

One unexpected and troublesome finding noted in Tables VII and VIII was the random survival of control mice (12.7% in Table VII and 12.1% in Table VIII). This resistance of control mice to challenge with 25 MLD of F. tularensis SCHU S5 cannot be attributed to loss of virulence of the challenge strain or experimental error. Most groups of AKR/J mice at this time had random intercurrent infections, during either the holding or experimental period. Current investigations by AR Division personnel indicate that the AKR/J mouse colony is infected with Sendai virus. Since a form of nonspecific protection against IP challenge with F. tularensis SCHU S5 has been demonstrated with 3 antigenically dissimilar bacterial antigens, it must be considered that a concurrent Sendai virus infection, a common problem in breeding colonies from October through April, may also afford some measure of nonspecific protection in control mice.

During this same time frame, an experiment was conducted to assess the length of time that killed Y. pestis antigen could potentiate the protective effect of ISC in syngeneic recipients challenged IP. Spleen cells from LVS-LVS immunized donors were transferred in the usual manner on day 9 post-booster to syngeneic recipients. Some recipients at time of transfer were inoculated IP with 10^7 killed SCHU S4 or $10^6 - 10^8$ killed Y. pestis antigen. Groups were challenged on days 1, 7, 14 or 21 postbooster. Survivors were backchallenged 30 days following primary challenge (Table VIII).

TABLE VII. SPECIFICITY OF ANTIGENIC REQUIREMENT FOR ADOPTIVE TRANSFER OF IMMUNITY TO AKR/J RECIPIENTS CHALLENGED IP 1 AND 7 DAYS FOLLOWING ISC TRANSFER.^a

KILLED ANTIGEN ^b	DOSE	RECIPIENT CHALLENGE		
		20 hr	7 days	30-day backchallenge
		(27 MLD SCHU S5) (IP)	(21 MLD SCHU S5) (IP)	(142 MLD SCHU S4) (SC)
<u>F. tularensis</u> SCHU S4	10^7	5/7 ^c	7/8	12/12
<u>B. pertussis</u> (DPT vaccine)	10^6	2/10	0/10	0/2
	10^7	0/10	7/10	0/7
	10^8	5/10	7/10	1/12
<u>S. typhi</u> (Typhoid vaccine)	10^6	4/10	2/10	0/6
	10^7	6/10	7/10	1/13
	10^8	3/10	6/10	1/9
None	--	0/10	--	--
<u>CONTROLS</u>				
Challenge	--	1/5	3/5	0/4
Normal spleen cells plus				
<u>B. pertussis</u>	10^8	0/10	3/10	0/3
<u>S. typhi</u>	10^8	0/10	2/10	0/2
<u>Antigen</u>				
<u>B. pertussis</u>	10^8	0/10	3/10	0/3
<u>S. typhi</u>	10^8	0/10	2/10	0/2

^a Donor vaccinated with 10^3 LVS SC and boosted 54 days later with 10^6 LVS IP; 10^8 spleen cells transferred 9 days postbooster.

^b B. pertussis antigen: washed DPT vaccine; S. typhi antigen: washed typhoid vaccine; F. tularensis antigen: phenol-killed and washed F. tularensis SCHU S4. Antigens contained in 0.2 ml of saline were administered IP to recipients at time of cell transfer.

^c Survivors/total.

TABLE VIII. SPECIFICITY OF ANTIGENIC REQUIREMENT FOR ADOPTIVE TRANSFER OF IMMUNITY TO AKR/J RECIPIENTS CHALLENGED IP FOLLOWING ISC TRANSFER.^a

KILLED ANTIGEN ^b	DOSE	SCHU S5 CHALLENGE POST ISC TRANSFER BY DAYS				
		1 (24 MLD)	7 (29 MLD)	14 (30 MLD)	21 (26 MLD)	30 Back- challenge ^c
None	--	1/9 ^d	0/9	1/9	1/9	0/3
<u>F. tularensis</u>	10^7	7/9	7/9	4/9	2/9	5/20
<u>Y. pestis</u>	10^6	2/9	0/9	0/9	0/9	0/2
	10^7	7/9	5/9	0/9	1/7	0/13
	10^8	1/9	7/9	3/9	0/9	1/11
<u>CONTROLS</u>						
Challenge	--	1/5	0/5	0/5	0/5	0/1
Normal spleen cells plus						
<u>F. tularensis</u>	10^8	0/9	4/9	1/9	0/9	0/5
<u>Y. pestis</u>	10^8	0/9	1/9	0/9	0/9	0/1
Antigen						
<u>F. tularensis</u>	10^8	0/6	2/6	2/6	2/6	0/6
<u>Y. pestis</u>	10^8	0/6	2/6	2/6	0/6	0/4

^a Donors vaccinated with 10^3 LVS SC and boosted 32 days later with 10^6 LVS IP; 10^8 spleen cells transferred 9 days postbooster.

^b Y. pestis antigen: washed Cutter vaccine; F. tularensis antigen: phenol-killed and washed F. tularensis SCHU S4. The antigens contained in 0.2 ml of saline were administered IP to recipients at time of ISC transfer.

^c Subcutaneous challenge with 120 - 130 MLD F. tularensis SCHU S4.

^d Survivors/total.

Data for Y. pestis-treated recipients confirm and expand those reported in Table V. The gradual loss of resistance over a 21-day period tends to support the nonspecific nature of the potentiation effected by this antigen.

One unexpected result was the decreased effectiveness of the F. tularensis SCHU S4 antigen; this result is in direct contradiction to previous experiments in which this antigen provided solid protection against SC challenge for at least 30 days after spleen cell transfer. In addition, only 5 of 20 survivors from primary challenged recipients of immune spleen cells and SCHU S4 antigen survived SC backchallenge with 10^2 SCHU S4; this contrasts with previous experience of nearly 100% survival. A possible explanation for these results might be that nonspecific protection afforded by concurrent Sendai virus infection in some mice could have prevented proliferation of the initial challenge strain in recipient macrophages, thus leaving the survivors more susceptible to 30-day backchallenge.

The data presented here demonstrate that the tularemia model in the AKR/J mouse provides an excellent system for study of cell-mediated and humoral immunity with evaluation in terms of animal survival to challenge with an extremely virulent organism. It was shown that the narrow protective ranges noted in other systems utilizing Listeria, Salmonella or tuberculosis, were most likely due to clearance of viable organisms in the donor spleen. In the tularemia system we have demonstrated that the protective effect could be greatly extended and enhanced by the use of killed specific antigen.

II. Attempts to induce effective, nonspecific host resistance (NSR) against lethal bacterial infections by vaccines currently in use, and to correlate host NSR with changes in phagocyte populations or delayed type hypersensitivity (DTH) have continued. Preliminary evidence¹ indicated that treatment of AKR/J mice with F. tularensis, strain LVS, or diphtheria-pertussis-tetanus toxoid vaccine (DPT), or 7.6% Na caseinate induced NSR against lethal infection with Listeria monocytogenes, strain Mack, or Salmonella typhimurium, strain Keller var. Copenhagen. The time of appearance of NSR following LVS vaccination correlated with mobilization of activated phagocytic cells, but not with the appearance of specific DTH. The present study is an expansion of this work.

Groups of AKR/J mice treated with LVS, DPT or 7.6% Na caseinate and appropriate control groups were challenged IP with lethal doses of L. monocytogenes (10^5 organisms) or S. typhimurium (10^3 organisms) at various intervals (1-60 days) after treatment. Results of Listeria challenges are shown in Table IX. These data confirm that NSR against L. monocytogenes in LVS vaccinees appeared as early as day 3, protected most animals on days 6-12, persisted in ~50% of mice through day 30, but was no longer detectable on day 60. Vaccination with DPT produced an even earlier response, protecting most animals within 48 hr and all animals on days 6-12, but by day 20 protection was no longer evident. Vaccination with a combination of LVS + DPT provided mice the earlier NSR induced by DPT and the later protection induced by LVS. Treatment with 7.6% Na caseinate solution IP induced NSR

in ~50% of the mice on days 3-9, but was ineffective at earlier or later times. Combination of LVS vaccination with Na caseinate treatment was no more effective than LVS vaccination alone.

TABLE IX. NONSPECIFIC RESISTANCE IN AKR/J MICE INDUCED BY VARIOUS TREATMENTS AGAINST LISTERIA MONOCYTOGENES (MACK).

TREATMENT	NO. SURVIVORS/NO. CHALLENGED BY POSTTREATMENT DAY								
	1	2	3	6	9	12	20	30	60
Control	1/18	0/12	0/24	0/18	2/18	1/24	1/18	0/30	1/18
LVS vaccine (10^2 SC)	0/18	2/18	6/18	10/12	11/12	12/12	6/12	7/18	1/12
DPT vaccine (0.1 ml IM)	1/12	8/12	16/18	12/12	12/12	12/12	1/12	0/12	3/12
LVS + DPT (10^2 SC) (0.1 ml IM)	3/12	5/18	6/12	12/12	12/12	12/12	4/12	6/12	0/12
7.6% Na caseinate (2 ml IP)	0/12	0/12	7/12	8/12	6/12	0/12	0/12	3/12	0/12
7.6% Na caseinate + LVS vaccine (2 ml IP) (10^2 SC)	0/12	0/12	7/12	12/12	12/12	12/12	8/12	7/12	2/12

S. typhimurium, strain Keller, was much more virulent for AKR/J mice than L. monocytogenes, strain Mack, but, unlike the Listeria strain, it readily became attenuated when monthly transfers on blood agar base slants were stored at 4 C, or when lyophilized cultures were stored at -20 C. Problems caused by attenuation were resolved by obtaining new cultures from Prof. S. Vas, McGill University, and storing the lyophilized cultures at -60 C. Results of Salmonella challenges are shown in Table X. Surprisingly, vaccination with LVS or DPT induced protection against S. typhimurium earlier and in more mice than was observed against L. monocytogenes. Almost all mice vaccinated with DPT were protected against lethal infection on days 1-9, but few remained protected by day 12. Vaccination with LVS, or LVS + DPT, was not as effective as DPT vaccine alone for protection on days 1-9, but was much more effective than DPT alone from days 12-30. Treatment with 7.6% Na caseinate did not induce NSR against lethal S. typhimurium infection, and in

combination with LVS vaccination was somewhat less effective than vaccination with LVS alone.

TABLE X. NONSPECIFIC RESISTANCE IN AKR/J MICE (n = 12) INDUCED BY VARIOUS TREATMENTS AGAINST SALMONELLA TYPHIMURIUM (KELLER).

TREATMENT	NO. SURVIVORS/NO. CHALLENGED BY POSTTREATMENT DAY								
	1	2	3	6	9	12	20	30	60
Control (n = 6)	1	0	0	0	0	0	0	0	2
LVS vaccine (10^2 SC)	4	9	9	10	8	10	5	5	7
DPT vaccine (0.1 ml IM)	11	11	11	10	12	4	0	0	4
LVS + DPT (10^2 SC) (0.1 ml IM)	3	7	6	9	11	10	3	7	2
7.6% Na caseinate (2 ml IP)	0	1	0	0	0	2	3	1	5
7.6% Na caseinate + LVS vaccine (2 ml IP) (10^2 SC)	0	9	11	11	9	10	6	4	0

It should be noted in reference to previously reported responses of mice to LVS vaccination that NSR against S. typhimurium was evident in most mice at least 24 hr before an increase was detected in the splenic phagocyte or nonspecific bactericidal peritoneal macrophage (BPM) populations, and 6 days before specific DTH appeared.¹ Protection against both L. monocytogenes and S. typhimurium persisted in mice long after the BPM population returned to normal. Furthermore, treatment with 7.6% Na caseinate by IP injection is known to induce a marked increase in the number of BPM and neutrophils within 24 hr;⁵ yet such treatment only induced NSR against L. monocytogenes in some mice after 72 hr, and was ineffective against S. typhimurium. Therefore, it is concluded that the time of appearance of host NSR did not correlate with mobilization of activated phagocytic cells nor with the appearance of specific DTH.

The results confirm the impression that effective host resistance to infectious diseases of military significance may be induced rapidly and nonspecifically by vaccines currently in medical use. Studies on the spectrum, duration, recall and enhancement of host NSR are continuing.

Presentation:

Little, S. F., H. T. Eigelsbach, and H. G. Dangerfield. Effectiveness of antibiotics for therapy of murine tularemia. Presented, 15th Intersci. Conf. Antimicrobial Agents Chemotherapy, 24-26 Sep. 1975 (Program, no. 309).

Publication:

Eigelsbach, H. T., D. H. Hunter, W. A. Janssen, H. G. Dangerfield, and S. G. Rabinowitz. 1975. Murine model for study of cell-mediated immunity: protection against death from fully virulent Francisella tularensis infection. Infect. Immun. 12:999-1005.

LITERATURI CITED

1. U. S. Army Medical Research Institute of Infectious Diseases, 1 July 1975. Annual Progress Report, FY 1975. pp. 202-207, Fort Detrick, Frederick, MD.
2. Eigelsbach, H. T., D. H. Hunter, W. A. Janssen, H. G. Dangerfield, and S. G. Rabinowitz. 1975. Murine model for study of cell-mediated immunity: protection against death from fully virulent Francisella tularensis infection. Infect. Immun. 12:999-1005.
3. Elberg, S. S., P. Schneider, and J. Fong. 1957. Cross-immunity between Brucella melitensis and Mycobacterium tuberculosis. Intracellular behavior of Brucella melitensis in monocytes from vaccinated animals. J. Exp. Med. 106:545-554.
4. Mackaness, G. B. 1970. Cell-mediated immunity to infection. Chapter 5, pp. 45-54. In Immunobiology (R. A. Good and D. W. Fisher, ed.). Sinauer Associates, Inc., Stamford, CT.
5. U. S. Army Medical Research Institute of Infectious Diseases, 1 July 1973. Annual Progress Report, FY 1973. pp. 91-96, Fort Detrick, Frederick, MD.

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27. TECHNICAL OBJECTIVE, ^j 28. APPROACH, 29. PROGRESS (Provide text of each with Security Classification Code.) 23 (U) Evaluate factors affecting development, persistence and expression of in vitro reactivity to antigenic stimulation of peripheral lymphocytes from immunized subjects and relate cellular response to host resistance against tularemia infections. This work unit is aimed at improving medical abilities to immunize troops against this potentially important BW agent. 24 (U) Isolate antigens from Francisella tularensis that can induce a specific proliferative response in sensitized lymphocytes. Employ selected preparations to examine the temporal course of development and duration of lymphocyte responsiveness as related to resistance in immunized and/or convalescent monkeys. 25 (U) 75 07 - 76 06 - Tularemia in the AKR/J mouse appeared to constitute the best model for relating in vitro responses of sensitized peripheral lymphocytes (PL) to specific resistance of the donor. Unlike primate PL, cultures with 100 thousand murine PL failed to exhibit antigen-specific transformation under conventional cultural conditions. Analysis of cultural requirement with splenic mononuclear leukocytes (SML) demonstrated that (1) 10% fetal calf serum was the best medium supplement, (2) nonspecific lymphocyte stimulation by the medium and other unknown factors increased linearly with culture density and interfered with demonstration of specific responses, (3) sensitive detection of antigen-specific responses could be achieved with 150,000 or more SML, incubation for 4 days and SML: killed antigen ratios of 1:1 - 1:4, and (4) the more highly sensitized the SML, the lower the antigen requirement. Findings for murine SML will be applied to culture of murine PL. No further work is planned at this time.						
29. Available to contractors upon originator's approval.						

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BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 105: Immune Responses of Peripheral Leukocytes

Background:

Defense of the host against certain microbial infections appears to reside primarily in the capability of the individual to mobilize macrophages and specifically sensitized lymphocytes. Although a number of in vitro correlates for this cell-mediated immune response have been proposed, an appropriate experimental model is required to evaluate the feasibility of utilizing in vitro responses of sensitized lymphocytes as a measure of the specific resistance of the lymphocyte donor. Published reports suggested that responses of Macaca mulatta to tularemia immunization/infection could be employed for this purpose. Classical tularemia antigens, i.e., Foshay, ether-extractable antigen (EEA) and phenol-extractable polysaccharide (CHO), and 5 fractions from late log-phase filtrates of Francisella tularensis SCHU S4 were prepared.¹ All antigens evoked specific transformation of peripheral mononuclear leukocytes (PML) from sensitized humans, but the reactions were suppressed by antibody-positive human serum. With PML from groups of control and tularemia-survivor monkeys, only Foshay and EEA reactions clearly identified all the survivors; CHO reactions showed no between-group differences; and some filtrate fractions identified high- and low-responder groups of survivors. Specific lymphocyte responsiveness persisted for at least 2 yr and was the same order of magnitude as that of lymphocytes from skin-test positive, immunized at-risk laboratory personnel.

Progress:

Relatively large groups of animals will be required to evaluate the relationship between antigen-induced lymphocyte responsiveness and resistance of the individual because of variability between individual responses to antigenic stimuli and the multiplicity of antigenic components capable of activating sensitized lymphocytes in vitro. A scarcity of monkeys and presumptive evidence for markedly increased resistance to tularemia in adult monkeys (i.e., nonvaccinated and vaccinated adult monkeys developed essentially the same signs of illness and survived challenge with fully virulent F. tularensis)² prompted search for another animal host. The AKR/J mouse, currently the model for adoptive transfer of cell-mediated immunity (CMI), was selected because: (1) it is the only inbred strain that, although highly susceptible to the virulent SCHU strains, is relatively resistant to SC inoculation with LVS vaccine, (2) relatively short-lived protection

against SCHU strains can be produced by LVS vaccine but not by killed vaccine or passive transfer of antiserum, and (3) splenocytes from LVS-vaccinated donors protect nonvaccinated syngeneic recipients against virulent challenge.³

Exploratory studies indicated that if cultures could be established at cell densities satisfactory for primate PML (10^5 PML/culture), it would be feasible to study the AKR/J model; since approximately 0.4 - 0.5 ml of cardiac blood ($2 - 2.5 \times 10^5$ PML) could be drawn apparently without harm to the donor, a single blood sample should contain a sufficient number of PML for 4-5 sets of replicate cultures. To investigate procedures for PML isolation and culture, pools of blood from AKR/J mice were employed. Ficoll-Hypaque separation procedures were less satisfactory for isolation of murine PML than for primate PML; yields of 70-80% primate PML were obtained consistently by centrifuging diluted blood at 250-300 g, while the highest yields of murine PML (60-66%) were achieved by centrifugation at 175 g. Moreover, in contrast to preparations of primate PML, murine PML suspensions had quite high erythrocyte contamination and 7-10% PMN cells.

Cultural studies were performed with pooled samples obtained 2-4 wk after LVS vaccination; diluted whole blood, washed cells from diluted whole blood or isolated PML were cultured with Foshay antigen (killed SCHU S4 cells), EEA or CHO in medium RPMI 1640 supplemented with 5 or 10% serum from nonvaccinated syngeneic donors. Although 80-90% of vaccinated donors should have been resistant to challenge with 10^3 SCHU S4, their PML failed to demonstrate significant proliferation with specific antigen. Thymidine incorporation for a representative experiment with 2-wk postimmunization PML and Foshay antigen are presented in Table I. The low counts contrast with counts previously

TABLE I. ^{14}C -THYMIDINE INCORPORATION BY 10^5 AKR/J PERIPHERAL LEUKOCYTES (DAY 14 POST-LVS, VACCINATION) CULTURED IN RPMI 1640 FOR 3, 4 OR 5 DAYS WITH PHENOL-KILLED *F. TULARENSIS* SCHU S4 ORGANISMS.

KILLED SCHU S4/ LYMPHOCYTE	GEOMETRIC MEAN ^{14}C CPM BY DAYS							
	Whole blood (1:10 dilution)		Washed cells from whole blood (1:10 dilution)			Cells isolated over Ficoll-Hypaque (10^5 cells/culture)		
	4	5	3	4	5	4	5	
None	19	33	19	22	50	18	48	
0.1	19	38	36*	86	38	19	33	
1.0	22	44	65	37*	36	25	28	
10.0	17	23	45	29	38	19	46	

* $P < 0.01$, compared to corresponding antigen-free cultures.

observed for 4-day cultures of isolated, immune primate PML, i.e., 1000-4000 CPM with tularemia antigens and 20-80 CPM in the absence of antigen. Marked variation between replicates and the few viable PML (< 20%) observed in 4- and 5-day cultures also indicated that cultural conditions were unsatisfactory.

Cell numbers required for definition of optimal conditions for cell separation of culture suggested use of a lymphocyte source other than blood. Consequently, studies were initiated with dissociated spleen cells. Spleens were minced with scissors, pressed through a 60-mesh screen, diluted in harvest fluid and passed several times through a 25-gauge needle. Approximately 5×10^8 splenic mononuclear leukocytes (SML)/spleen were recovered from vaccinated mice and 3×10^8 , from nonvaccinated mice. Harvests prepared in Hanks balanced salt solution or RPMI 1640 medium with 25 mM Hepes buffer consisted predominantly of 2-3-cell clumps of SML that sedimented with erythrocytes during Ficoll-Hypaque separation, resulting in recovery of 5-10% SML at the interface. Yields of isolated SML were somewhat improved by harvest with calcium-magnesium-free phosphate buffer, pH 7.4, but maximum recovery (35-40%) was achieved with a solution of 0.003 M KCl, 0.14 M NaCl, 0.01 M EDTA, 0.01 M phosphate and 0.5% bovine serum albumin. Suspensions of cells collected from the Ficoll-Hypaque interface after centrifugation at 200 g for 30 min and washed in harvest fluid were free of platelets, but contained some erythrocytes and 5-10% granulocytes. Viability, as indicated by trypan blue exclusion, was generally > 97%.

Responsiveness of lymphocytes to mitogenic effects of phytohemagglutinin (Bacto, PHA-P) was used as a guide to establish cultural conditions that would support lymphocyte transformation. A medium described in many publications on murine splenocytes, i.e., RPMI 1640 supplemented with 25 mM Hepes, 5% fetal calf serum (FCS) and 100 units penicillin and 100 μ g streptomycin/ml, was employed. The effect of culture density and PHA-P concentration was examined with 2-day cultures of isolated CD-1 mouse SML recovered at the interface or from the Ficoll-Hypaque layer (Table II). With 0.25 μ g PHA-P, lymphocyte transformation failed to occur; maximum proliferative effects were observed with 2.5 μ g PHA-P at cell densities of $2-5 \times 10^5$ /culture and SML recovered from the Ficoll-Hypaque layer were less reactive than those from the interface. Nonspecific incorporation of thymidine increased in direct proportion to culture density. When cultures of diluted whole blood and isolated SML from the same CD-1 mouse were compared, SML cultures proved to be markedly more responsive (Table III).

As compared to CD-1 splenocyte cultures, AKR/J splenocytes exhibited markedly greater nonspecific incorporation of thymidine. Therefore, the contribution of the serum supplement in the medium to nonspecific stimulation was investigated by comparing the effect of 5, 10 and 15% concentrations of FCS human or monkey serum on viability and PHA-response of AKR/J splenocytes. Although FCS was most active for induction of nonspecific

TABLE II. EFFECT OF CELL DENSITY ON THYMIDINE INCORPORATION BY CD-1 LEUKOCYTES RECOVERED FROM THE INTERFACE OR WITHIN THE FICOLL-HYPAQUE LAYER AFTER CULTURE FOR 2 DAYS WITH 2.5 μ g PHA-P.

LEUKOCYTE PREPARATION	PHA-P/ CULTURE (μ g)	GEOMETRIC MEAN ^{14}C COUNTS PER MINUTE						
		Leukocytes $\times 10^5$ /culture						
		1	2	4	5	6	8	10
Interface fraction (6 replicates)	None	25	56		396			
	0.25	28	58		377			
	2.5	99	801 ^a (14) ^a		9394 (24)			
Ficoll-Hytpaque layer (3 replicates)	None		32	106		296	466	606
	0.25		43	130		277	446	561
	2.5		95	147 ^b (14)		1872	4104	3259

a () Stimulation Index = CPM with PHA-P/CPM in absence of PHA-P, where P < 0.0001.

TABLE III. THYMIDINE INCORPORATION BY 2×10^5 CD-1 LEUKOCYTES FROM PERIPHERAL BLOOD OR SPLEEN AFTER CULTURE FOR 2 DAYS WITH 1 μ g PHA-P.

PHA-P/ CULTURE (μ g)	WHOLE BLOOD (1:10)		ISOLATED SPLENOCYTES	
	CPM	Stimulation Index	CPM	Stimulation Index
None	60		71	
1.0	102*	1.7	446*	6.3

* P < 0.0001.

stimulation, cultures with 10% FCS consistently exhibited the least variability between replicates and were most viable and responsive to PHA stimulation (Table IV).

TABLE IV. THYMIDINE INCORPORATION (CPM) AND VIABILITY OF AKR/J SPLENIC LEUKOCYTES (SML) AFTER CULTURE FOR 2 DAYS IN RPMI 1640 SUPPLEMENTED WITH 10% FETAL CALF SERUM, MONKEY OR HUMAN SERUM (MITOGENIC STIMULATION, 2.5 μ g PHA-P).

SERUM ADDITIVE	2.5 $\times 10^5$ SML/culture				5 $\times 10^5$ SML/CULTURE			
	CPM-day 2		Viability by day		CPM-day 2		Viability by day	
	PHA-P (μ g)		2	5	PHA-P (μ g)		2	5
	0	2.5			0	2.5		
None	16	22	17	ND ^a	2205	2085	48	ND
Fetal calf	1635	6246 (3.8) ^b	67	77	5489	7852 (1.4) ^c	74	74
Monkey	195	536	34	10	405	1910 (4.7) ^c	50	46
Human	286	1052	30	20	1081	4488	44	40

a Not done; few viable cells.

b () Stimulation Index: $\frac{\text{Geometric mean CPM with PHA-P}}{\text{Geometric mean CPM without PHA}}$; $P < 0.0001$.

c () Stimulation Index: $P < 0.001$.

In the absence of PHA-P, FCS-induced ^{14}C -incorporation by AKR/J splenocytes was affected by cell density and time of incubation (Table V). In 2-day cultures nonspecific activity increased linearly with increasing cell density, from ~ 75 CPM for 0.8×10^5 SML to 3200 CPM for 8×10^5 SML. At cell densities of $0.8 - 1.6 \times 10^5$, increased activity was observed in 3- and 5-day cultures; at somewhat higher cell densities, activity remained essentially the same for 5 days; but at cell densities of $6-8 \times 10^5$, decreased thymidine-incorporation was evident in 5-day cultures. Survival curves for cultures with $1.6 - 8.0 \times 10^5$ SML were almost identical; 20-35% of cells became nonviable within 24 hr, but surviving cells remained healthy for 5 days. With fewer SML, a similar initial loss was observed but viability continued to decrease with longer incubation time.

TABLE V. EFFECT OF CULTURE DENSITY AND INCUBATION TIME ON VIABILITY AND NONSPECIFIC INCORPORATION OF THYMIDINE (CPM) BY AKR/J SPLENIC MONONUCLEAR LEUKOCYTES (SML).

SML x 10 ⁵ / CULTURE	CPM BY DAY			VIABILITY BY DAY			
	2	3	5	1	2	3	5
0.8	76	123	297	68	48	34	33
1.6	124	445	793	66	55	62	70
3.2	679	681	806	68	59	56	70
4.8	1055	1136	1340	75	70	66	64
6.4	2249	2876	1967	76	62	60	61
8.0	3178	3421	2336	79	71	74	62

In parallel cultures incubated with 2.5 µg PHA-P, 2- and 3-day values for cultures with $0.8 - 4.8 \times 10^5$ SML indicated significant levels ($P < 0.001$) of mitogen-induced proliferation; although thymidine incorporation increased in cultures with greater cell densities, differences between PHA-P and PHA-P-free cultures were less marked because of high background counts and variability between replicates. The highest stimulation indices (14-21) were observed in 3-day cultures with $0.8 - 1.6 \times 10^5$ SML. Thymidine incorporation was decreased significantly ($P < 0.001$) in 5-day cultures and was essentially the same as that observed in PHA-P-free cultures.

At the time of these studies, there was high incidence of persistent illness in AKR/J mice apparently attributable to Sendai virus infection in the breeding colony; thus responses of "healthy" mice to LVS vaccination and SCHU challenge became less predictable. When inbred mouse strains RF/J and SWR/J were examined as potential alternative models for adoptive transfer studies, PHA-P-induced proliferation of their splenic SML was investigated (Table VI). Yields of isolated splenocytes from RF/J and SWR/J mice were somewhat lower than from AKR/J mice. The amount of nonspecific stimulation observed with 2-day cultures appeared to be strain-related, but there was little difference between strains with respect to stimulation indices for PHA-P cultures. Viability of lower density cultures (2.5×10^5 SML) from all strains showed little change between day 2 and day 5 in the absence of PHA-P but was markedly reduced after culture for 5 days with PHA-P, suggesting that the previously noted absence of PHA-P stimulation in 5-day AKR/J cultures may reflect a loss of viability.

TABLE VI. PHA-P-INDUCED THYMIDINE INCORPORATION (CPM) AND VIABILITY OF ISOLATED SPLENOCYTES (SML) FROM AKR/J, RF/J OR SWR/J MICE AFTER CULTURE FOR 2 DAYS IN RPMI 1640 SUPPLEMENTED WITH 10% FETAL CALF SERUM.

Strain	% Yield	ISOLATED SPLENOCYTES	PHA-P/ CULTURE (μ g)	2.5×10^5 SML/CULTURE			5.0×10^5 SML/CULTURE		
				CPM Day 2	Viability		CPM Day 2	Viability	
				Day 2	Day 2	Day 5	Day 2	Day 2	Day 5
AKR/J	30	None	292	42	36		2665	51	75
		2.5	1670 (5.7) ^a	40	19		7643 (2.9)	28	16
RF/J	22	None	927	62	52		4315	53	68
		2.5	4332 (4.7)	60	36		11797 (2.7)	32	21
SWR/J	24	None	300	58	72		1228	74	78
		2.5	1264 (4.2)	69	38		4380 (3.6)	40	54

a () Stimulation Index; P <0.0001.

For investigation of antigen-specific responses, dissociated spleen cells from normal AKR/J mice or from LVS-vaccinated AKR/J mice were employed; donors were approximately 20-wk old and comparison studies were performed with mice from the same shipment. Foshay antigen prepared from strain SCHU S4 and dialyzed exhaustively against culture medium was selected because in previous studies this antigen proved to be highly effective for stimulation of lymphocytes from vaccinated primates. In most trials, antigen-free cultures had high background counts and addition of antigen effected only 2-3-fold increases in thymidine incorporation by sensitized lymphocytes. As in previous studies, the FCS supplement was responsible for some of the high background activity, and there was some evidence that a difference between batches of medium RPMI 1640 contributed to the background. More recent trials indicate that although FCS and medium batch are additive contributory factors, some as yet unknown variable exerts a greater influence on background count.

Sensitive detection of antigen stimulation was possible only in the presence of low background stimulation. As with PHA-P, the concentration of splenocytes or antigen and time of incubation affected detection of antigen-specific stimulation. When postbooster SML cultures were harvested at 3, 4, 5 or 6 days, antigen-specific proliferative responses (P < 0.001)

were detected in cultures with $4-6 \times 10^5$ SML only on day 3, in those with 2×10^5 SML on days 3 and 4 and with 1×10^5 SML on days 4 and 5. By day 6 there was a 5-10-fold reduction in thymidine incorporation by all cultures and there was no evidence for antigen stimulation. Proliferative response could not be related to the number of viable SML because at culture densities $> 2 \times 10^5$, the % viable splenocytes remained essentially the same throughout the experimental period.

Antigen-induced incorporation of thymidine by 4- and 5-day cultures from nonvaccinated mice, from vaccinated mice 73 days after SC inoculation with 10^3 LVS organisms and from vaccinees 9 days after IP booster with 10^6 LVS organisms is presented in Table VII.

TABLE VII. INCORPORATION OF ^{14}C -THYMIDINE BY 4- AND 5-DAY CULTURES OF SPLENOCYTES OBTAINED FROM NORMAL AKR/J MICE, 73 DAYS AFTER LVS PRIMARY VACCINATION OR 9 DAYS AFTER LVS BOOSTER OF VACCINATED MICE.

SPLENOCYTES/ CULTURE ($\times 10^5$)	KILLED SCHU S4/ CULTURE ($\times 10^5$)	^{14}C -THYMIDINE CPM					
		Normal Cells		Vaccinee Cells		Postbooster Cells	
		Day	Day	Day	Day	Day	Day
0.5	--	43	67	56	29	61	48
	0.2	58	56	67	30	106*	118
	2.0	51	39	61	34	149	142*
	20.0	73	78	44	54	181	287
1.0	--	95	70	47	46	66	51*
	0.2	120	107	67	62*	297*	460*
	2.0	89	88	91	119*	632*	731*
	20.0	155	133	130	669*	996	950
1.5	--	126	174	73	43	99*	87*
	0.2	103	376	163*	109*	952*	1246*
	2.0	194*	410	244*	710*	2450*	1704*
	20.0	267	549	640	909	2602	3299
2	--	265	677	76	308	277	652
	0.2	200	738	214*	1018	1565*	3338
	2.0	280	567	350*	1479	2532*	4476
	20.0	513	1248	1050	2595	2971	5808

* $P < 0.001$, compared to corresponding antigen-free culture.

These data indicated that: (1) incubation of cultures for 4 days at SML:organism ratios of 1:1 - 1:4 permitted the most sensitive detection of antigen-specific stimulation, (2) highly sensitized SML obtained after a booster injection were markedly more responsive than comparable SML from 73-day vaccinees and reacted at lower SML:organism ratios (1:0.1 - 1:0.2), and (3) in 5-day cultures with 2×10^5 SML, variability in background counts prevented evaluation of antigen stimulation. Although these data are highly encouraging and emphasize the need for definition and elimination of conditions that enhance nonspecific stimulation of murine lymphocytes, no further work is planned and this work unit will be terminated.

Publications:

None.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1975. Annual Progress Report FY 1975. p. 217-224. Fort Detrick, Frederick, MD.
2. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1973. Annual Progress Report FY 1973. p. 215-220. Fort Detrick, Frederick, MD.
3. Eigelsbach, H. T., D. H. Hunter, W. A. Janssen, H. G. Dangerfield, and S. G. Rabinowitz. 1975. Murine model for study of cell-mediated immunity: protection against death from fully virulent Francisella tularensis infection. Infect. Immun. 12:999-1005.

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31. TECHNICAL OBJECTIVE, 32. APPROACH, 33. PROGRESS (Provide individual paragraphs identified by number. Proceed with Security Classification Code.) 23 (U) Devise more efficient methods for treating and monitoring the effects of treatment of respiratory bacterial infections. This information is essential for the expeditious therapy of troops subsequent to BW attack. 24 (U) After obtaining data on feasibility of aerosol therapy, determine pharmacodynamics, toxicity, and efficacy. 25 (U) 75 07 - 76 06 - High concentrations of kanamycin were found in lungs of rats for at least 96 hr and in spleen for at least 24 hr after aerosol administration, and in kidneys (72 hr) and urine (2 hr) after intramuscular (IM) injection. Kanamycin disappeared from blood within 40 min regardless of route of administration. Klebsiella pneumoniae infection increased the amount of antibiotic in lungs and urine after IM and in spleen after IM and aerosol treatment. Following respiratory Klebsiella infection the levels of plasma lysozyme, alpha-2-macroglobulin, zinc and albumin are markedly altered suggesting that these biochemical parameters may be useful indicators of severity of infection. Aerosols of ribavirin given continuously or intermittently (90 min/day) eliminate such signs of influenza in squirrel monkeys as coughing and coryza. They do not affect systemic signs such as leukopenia or increased respiratory rate. Publications: Antimicrob. Agents Chemother. 8:585-590, 1975. J. Infect. Dis. 132:689-693, 1975. Antimicrob. Agents Chemother. 10: in press, 1976 Program, 15th Intersci. Conf. Antimicrob. Agents Chemother., No. 310, 1975.							

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 109: Aerosol Therapy of Respiratory Bacterial Infections

Background:

We have reported that aerosols of kanamycin were more effective for the therapy of respiratory Klebsiella pneumoniae infection in mice than IM injection¹ and have presented studies of the kinetics of kanamycin clearance in rats after treatment. Kanamycin persists in the lungs after aerosol treatment and in the kidneys after IM. Preliminary observations of the changes in induced metabolic sequelae (IMS) in rats after Klebsiella infection have been outlined.¹

Utilizing aerosols of kanamycin for the treatment of Klebsiella infection in mice, it was found that aerosolized drug was mainly effective in the lungs and bacteremia was affected, if a pulmonary focus existed, but not if the bacteremia arose from an extrapulmonary source.²

In this report we present additional data on clearance of kanamycin from both normal and Klebsiella-infected rats and information gained from a continuing investigation of IMS (collaborative project with MAJ Powanda, Work Unit No. 834 01 401).

An additional subject for experimentation has been the therapy of influenza virus infection in squirrel monkeys with aerosols of ribavirin or rimantadine (collaborative project with MAJ Stephen, Work Unit No. 834 02 411). Effective use of these compounds in aerosols for the treatment of influenza virus-infected mice as well as the primate model have been reported.^{3,4}

Lastly, we present data on the use of radiolabeled gallium citrate (⁶⁷Ga) as a possible indicator of severity of respiratory infection.

Progress:

As a preliminary to subsequent clearance studies, an experiment was performed to determine the nonspecific antimicrobial activity of selected tissues of infected and normal rats. Homogenates of lungs, spleen and kidney had activity equal to approximately 1.0 µg/gm of kanamycin whether infection was present or not. Two additional experiments were then performed to determine the binding capacity of selected tissues for the antibiotic.

The binding capacity of the tissue was calculated as the % of kanamycin in the washed pellet relative to that in the original homogenate (Table I). Small amounts of kanamycin were found by lung, spleen and kidney, a moderate amount by the liver, and none by blood. In vivo binding was carried out by assaying homogenates and washed tissues from both infected and normal rats that had been given 12 mg/kg of kanamycin by aerosol or IM routes 24 hr earlier. No effect of infection was observed (Table II). Noteworthy here was the absence of kanamycin in the liver in vivo despite the binding capacity of this organ in vitro (Table I).

TABLE I. IN VITRO BINDING OF KANAMYCIN (12 µg/ml) BY RAT-TISSUE HOMOGENATES^a

TISSUE	% BOUND
Blood	0
Lung	15
Liver	39
Spleen	10
Kidney	15

TABLE II. IN VIVO BINDING OF KANAMYCIN (12 mg/kg BODY WT) IN NORMAL AND K. PNEUMONIAE-INFECTED RAT TISSUES

TISSUE	% ANTIBIOTIC FOUND AT 24 HR			
	Aerosol		IM	
	Normal	Infected	Normal	Infected
Blood	0	0	0	0
Lung	10.1 ^a	17.4 ^a	trace	trace
Liver	0	0	0	0
Spleen	trace	trace	trace ^a	trace ^a
Kidney	0	trace	43.3	29.4 ^a

^aNo significant difference (Student's t test).

Persistence of kanamycin in lungs after aerosol administration and in kidneys after IM administration is shown in Table III. Also of interest in the experiment was the rapid disappearance of antibiotic from the blood regardless of method of administration.

TABLE III. KANAMYCIN CONCENTRATION IN SELECTED TISSUES OF NORMAL AND RESPIRATORY KLEBSIELLA PNEUMONIAE-INFECTED RATS^a

ROUTE OF TREATMENT	HR AFTER TREATMENT	CONC. OF KANAMYCIN ($\mu\text{g}/\text{gm}$ or ml)					
		Normal			Infected		
		Blood	Lung	Kidney	Spleen	Lung	Kidney
IM Aerosol	0.5	9 1	2 13	6 1	1 2	6 62	33 1
IM Aerosol	24	0 0	1 4	4 1	1 3	1 17	8 1
IM Aerosol	48	0 0	1 4	4 1	1 1	1 15	9 1
IM Aerosol	72	b b	b b	b b	0 1	2 6	8 1

^a Kanamycin dose = 20 mg/kg.

^b Not done.

An experiment was carried out in which the effect of infection was investigated during the first 330 min following administration of the antibiotic (kanamycin was administered 24 hr postinfection). Data are presented in Table IV. A marked effect of infection was noted in the urine and lungs of rats given IM inoculation and in the spleens after administration by both routes.

Because kanamycin persisted in lungs after aerosol therapy, an experiment was performed to ascertain whether it was bound to pulmonary tissues or free in the lumen of the airways. Rats were killed 24 hr after therapy, and the trachea and bronchi were vigorously washed. No kanamycin was recovered, suggesting that the antibiotic was bound or was sequestered intracellularly.

The antiviral studies in squirrel monkeys have shown that continuously administered aerosols of ribavirin given 6 or 24 hr after infection effectively alter those signs of disease that result from tracheo-bronchial irritation such as sneezing, coughing and coryza, but do not affect signs of systemic origin such as increased respiratory rates or leukopenia. An example of these differences is seen in Table V in which monkeys were given continuously disseminated aerosols of ribavirin at a rate of 7 mg/kg/day for 4 days.

When an even smaller dose, 3.6 mg/kg/day, was administered for 80 min/day

TABLE IV. EFFECT OF *K. PNEUMONIAE* INFECTION ON CLEARANCE OF KANAMYCIN FROM RATS

TIME AFTER TREATMENT (min)	ROUTE	CONCENTRATION OF KANAMYCIN ($\mu\text{g}/\text{organ or ml}$)					
		Lungs		Kidneys		Urine	
		Infected	Normal	Infected	Normal	Infected	Normal
40	IM Aerosol	7	6	84	44	3500	307
		66	86	2	2	26	99
120	IM Aerosol	25	5	65	21	873	294
		45	56	2	3	52	167
240	IM Aerosol	17	8	23	24	a	174
		33	39	2	2	a	100
330	IM Aerosol	19	5	26	23	a	69
		29	25	5	2	a	186

^aNo urine sample obtained, output too low.

TABLE V. EFFECT OF CONTINUOUSLY-DISSEMINATED RIBAVIRIN^a ON INFLUENZA IN SQUIRREL MONKEYS

INFECT.	% OF MONKEYS COUGHING		% OF MONKEYS WITH CORYZA		LEUKOCYTES/ mm ³ (x10 ³)		RESP. RATE (breaths/min)	
	Cont	Trtd	Cont	Trtd	Cont	Trtd	Cont	Trtd
-1	0	0	0	0	9.0	8.3	100	106
+1	67	50	67	0	11.4	11.9	113	125
2	100	75	100	25	6.3	5.7	126	123
3	100	25	100	75	5.8	4.9	137	144
4	100	0	100	0	3.7	5.2	150	148
5	100	0	100	25	5.7	5.4	159	146
6	100	0	100	25	5.7	4.8	146	157
7	67	0	100	25	10.0	5.7	132	157
8	0	0	100	25	15.9	9.7	114	117
12	0	0	100	25	12.3	16.5	100	109

^aDose was 7 mg/kg/day for 4 days.

(intermittent aerosol) the drug effectively stopped coughing and sneezing. When 7.5 mg/kg was administered daily by IM injection, however, no therapeutic effect was noted. The same injected dose of rimantadine was also ineffective.

A few treated monkeys died during the course of these experiments and some evidence of liver damage was seen. Therefore, 8 monkeys were exposed to 40 mg/kg/day of ribavirin for 4 days by continuous aerosol. Four were killed and necropsied at 4 days; 4 were killed at 25 days; 4 monkeys, given aerosols of distilled water for 4 days, served as controls. Laboratory tests at 1, 5, 9 and 25 days included WBC and RBC counts, hematocrit, hemoglobin, weights, reticulocyte concentrations, SGOT and β -glucuronidase determinations. No laboratory evidence of toxicity was observed. The only blood changes were those due to frequent sampling. Histopathological examination yielded equivocal results. Although a higher incidence of fatty metamorphosis of the liver seemed to be present in drug-treated monkeys at 4 days but not 25 days, the numbers were insufficient to provide conclusive evidence. This experiment will be repeated.

A single experiment was performed with continuously-disseminated aerosols of rimantadine. The drug appeared to irritate the respiratory tract of exposed monkeys, thus making evaluation of therapeutic effectiveness almost impossible. The problem of respiratory irritation will be investigated. Meanwhile, the use of rimantadine for intermittent therapy (90 min/day) will be tried.

The results of investigations of IMS following K. pneumoniae challenge of rats indicate that the levels of plasma Zn, lysozyme, seromucoid, albumin, and α_2 -macrofetoprotein (α_2 -MFP) change after infection. Results further showed that threshold numbers of bacteria ($\sim 10^5$ organisms/gm of tissue) must exist in the lungs before α_2 -MFP, Zn, and lysozyme values depart significantly from those of the controls. These findings are discussed in detail in a paper that has been submitted for publication.⁵ The measurement of IMS following aerosol and IM therapy of infected rats with kanamycin is currently under investigation.

The possible use of ^{67}Ga citrate as an indicator of the severity of respiratory infection has been investigated by injecting mice IP 24 hr after respiratory K. pneumoniae-infection with 20 $\mu\text{Ci}/\text{kg}$ of ^{67}Ga citrate. The amount of isotope in selected tissues of normal mice was compared to that in infected mice. Also, the amount of isotope in the lungs of infected and normal animals was compared to that in muscle of the same mouse. These comparisons are given as ratios in Table VI. An experiment is planned in which mice will be injected with isotope at various stages of infection to determine whether severity of response correlates with degree of isotope incorporation.

TABLE VI. UPTAKE OF ^{67}GA CITRATE BY SELECTED TISSUES OF NORMAL AND K. PNEUMONIAE-INFECTED MICE

MIN AFTER ISOTOPE INJECTION	RATIO OF INFECTED/ NORMAL TISSUE			RATIO OF LUNG/MUSCLE	
	Lungs	Blood	Muscle	Infected	Normal
30	2.78	1.74	0.90	1.55	0.50
90	2.53	1.45	0.86	2.27	0.77
120	2.42	1.38	0.81	1.92	0.64
150	1.73	1.26	1.05	1.87	1.14
180	3.49	1.87	1.29	1.85	0.68

Future plans for this work unit, in addition to those already discussed, include quantitative comparison of aerosol-induced hypersensitivity reactions with those induced by ID sensitization utilizing guinea pigs initially, and primates as a confirmatory species. Development of a Pseudomonas aeruginosa animal model for use as a model respiratory system for melioidosis (Pseudomonas pseudomallei) will be carried out. This model will be used for evaluation of aerosolized antibiotics in the treatment of potential bacterial pneumonias resulting from possible biological warfare attack. The study of IMS in infected rats to establish new indices of therapeutic efficacy in the treatment of bacterial pneumonias will be continued. The primate confirmation work using aerosolized antiviral compounds for the treatment of viral pneumonia will be continued.

Presentation:

Berendt, R. F., and J. S. Walker. Fate of aerosols of kanamycin in normal and respiratory Klebsiella pneumoniae (Kpn) infected rats. Presented, 15th Interscience Conference on Antimicrobial Agents and Chemotherapy. Washington, D.C., 24-26 Sep 1975 (Abstracts, No. 310).

Publications:

1. Berendt, R. F., G. G. Long, and J. S. Walker. 1975. Treatment of respiratory Klebsiella pneumoniae infection in mice with aerosols of kanamycin. *Antimicrob. Agents Chemother.* 8:585-590.
2. Berendt, R. F., G. G. Long, and J. S. Walker. 1975. Influenza alone and in sequence with pneumonia due to Streptococcus pneumoniae in the squirrel monkey. *J. Infect. Dis.* 132:689-693.
3. Berendt, R. F., and J. S. Walker. 1976. Distribution of kanamycin in rat tissue after aerosol or intramuscular treatment. *Antimicrob. Agents Chemother.* 10: in press.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1975. Annual Progress Report, FY 1975, pp. 247-252. Fort Detrick, Frederick, MD.
2. Berendt, R. F., G. G. Long, and J. S. Walker. 1975. Treatment of respiratory Klebsiella pneumoniae infection in mice with aerosols of kanamycin. *Antimicrob. Agents Chemother.* 8:585-590.
3. Walker, J. S., E. L. Stephen, and R. O. Spertzel. 1976. The use of small-particle aerosols of antiviral compounds for the treatment of type A influenza pneumonia in animal models. *J. Infect. Dis.* 133(Suppl.):140-144.
4. Berendt, R. F., G. G. Long, and J. S. Walker. 1975. Influenza alone and in sequence with pneumonia due to Streptococcus pneumoniae in the squirrel monkey. *J. Infect. Dis.* 132:689-693.
5. Berendt, R. F., G. G. Long, P. G. Canonico, F. B. Abeles, and M. C. Powanda. 1976. Induced metabolic sequelae of respiratory Klebsiella pneumoniae in rats. (submitted for publication).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ¹	2. DATE OF SUMMARY ²	REPORT CONTROL SYMBOL ³
3. DATE PREV SUMMARY 75 12 11	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCY ⁴ U	6. WORK SECURITY ⁵ U	DA OF6423	76 07 01	DD-DR&E(AR)626
10. NO./CODES: ⁶ a. PRIMARY b. CONTRIBUTING c. CONFIDENTIAL CARDS 114(e)(f)	PROGRAM ELEMENT 62760A	PROJECT NUMBER 3A762760A834	7. REGRADING ⁷ NA	8. ORIGIN INSTRNM ⁸ NL	9. SPECIFIC DATA- TRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A WORK UNIT
				TASK AREA NUMBER 02	WORK UNIT NUMBER 110	
11. TITLE (Provide with Security Classification Code) (U) Therapeutic manipulation of metabolo-endocrine controls during sepsis						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁹ 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry						
13. START DATE 75 12	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house			
17. CONTRACT/GRAANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS		
20. DATES/EFFECTIVE:		EXPIRATION:	FISCAL YEAR	PREVIOUS CURRENCY	1.0	120
21. NUMBER: NA		22. AMOUNT: \$ CUM. AMT.	76	77	1.0	164
23. RESPONSIBLE DOD ORGANIZATION		24. PERFORMING ORGANIZATION		25. FUNDS (in thousands)		
NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		NAME: Physical Sciences Division USAMRIID ADDRESS: Fort Detrick, MD 21701				
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Provide name if U.S. Armywide Institution) NAME: Kaminski, M. V. TELEPHONE: 301 663-7181 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME:				
26. GENERAL USE Foreign intelligence considered		27. POC:DA				
28. REFERENCES (Provide name with Security Classification Code) (U) Hyperalimentation (U) BW defense; (U) Military medicine; (U) Parenteral nutrition; (U) Metabolic defects; (U) Infection; (U) Rhesus monkeys; (U) Rats						
29. TECHNICAL OBJECTIVE, 30. APPROACH, 31. PROGRESS (Provide individual paragraphs identified by number. Provide rest of area with Security Classification code.) 23 (U) Lessen or reverse the catabolic effects of infection-induced stress by infusing appropriate profiles of micronutrients and substrates. Effect of parenteral nutrition can be used as a means of eliminating the metabolic changes associated with disease in the physical manifestations of that disease or a prolonged recovery period. In addition to the relation of this project to BW attack the expertise gained through work in this area is immediately applicable to the soldier infected secondary to a war wound. 24 (U) Remove the pancreas, and if necessary, the distal small bowel, in order to have a glucagon-free subhuman primate. All monkeys will be fed parenterally; half will be given exogenous glucagon. Ability of these monkeys to withstand bacterial sepsis will be measured and attempts will be made to improve micronutrient forms of therapy. 25 (U) 75 12 - 76 06 - Metabolic consequences of septic and/or nonseptic stress were examined in 7 separate experiments using a 2 x 2 block design. It was shown that streptococcal infection initiates specific metabolic changes in rats which can be distinguished from those caused by trauma of long-bone fracture. The role of glucagon induced by septic stress was examined for the capacity of intact and pancreatectomized animals to convert radiolabeled alanine to glucose and octanoate to ketone bodies. Glucagon does play a significant role in these processes. To evaluate the metabolic consequences of amino acid versus dextrose containing parenteral maintenance solutions a study was conducted at WRGH. Amino acid infusions act as glucagon and growth hormone secretagogue and do not stimulate insulin. The combined effect results in a beneficial ketonemia, less negative nitrogen balance and improved protein sparing. Publications: Clin. Res. 24:501A, 1976. Surg. Forum, abstract in press, 1976, Am. J. Clin. Nutr., in press						

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 110: Therapeutic Manipulation of Metabolo-Endocrine Controls During Sepsis

Background:

Part I.

Little distinction between septic and nonseptic trauma has been made in descriptions of their metabolic consequences. However, a variety of inflammatory stresses in rats (bacterial sepsis, endotoxemia, or turpentine-abscess) either prevented or diminished starvation-induced ketosis.

Part II.

Glucagon is a recognized mediator inducing many of the metabolic changes associated with septic stress. Until the present time, evaluation of the in vivo role of glucagon has been impossible because of the presence of enteroglucagon. Although results are preliminary, it appears that surgical removal of the pancreas without evisceration will produce a suitable model in the rhesus monkey.

Part III.

Compared to 5% dextrose (5D) the postoperative beneficial ketosis and nitrogen sparing observed during 3.5% amino acid (AA) is accepted. The originally hypothesized metabolic mechanism of reduced serum insulin however, is difficult to justify since this alone neither produces or activates cAMP-dependent triglyceride lipase or hepatocyte carnitine nor increases protein sparing. In addition to providing nitrogen, amino acid infusions stimulate growth hormone (GH) and glucagon (G) secretion, whose combined actions produce ketosis and protein sparing.

Progress:

Part I.

To study metabolic changes commonly observed during infection and overlapping trauma, rats were deprived of food and used in 4 groups:

I, SC injection of 10^4 heat-killed *Streptococcus pneumoniae*; II, same as I plus femoral fracture (digital, noninvasive technique during anesthesia); III, same as II but 10^4 virulent *S. pneumoniae* injected; IV, 10^4 virulent organisms alone. There were no significant differences in the metabolic parameters studied between starvation or starvation plus femoral fracture. By 24 or 48 hr following infection, however, many nonseptic (Groups I and II) values were significantly different from septic (Groups III and IV) as shown in Table I.

TABLE I. METABOLIC VALUES IN NONSEPTIC AND SEPTIC STRESSES

PARAMETER		TIME (hr)	NONSEPTIC	SEPTIC	P
β -Hydroxybutyrate	$\mu\text{M}/\text{ml}$	48	0.74 ± 0.13	0.0 ± 0.0	<0.001
Free fatty acids	$\mu\text{Eq}/\text{L}$	24	661.00 ± 59.00	388.0 ± 54.0	<0.01
[^{14}C]-Alanine to glucose	dpm/ μL	48	14.60 ± 0.50	18.6 ± 0.5	<0.001
α_2 -Macrofeto-globulin	U/ml	48	2.00 ± 1.60	52.4 ± 9.6	<0.001
Ceruloplasmin	$\mu\text{g}/\text{dl}$	48	72.00 ± 3.0	108.0 ± 4.0	<0.001
Serum Zn	$\mu\text{g}/\text{dl}$	48	108.00 ± 4.0	27.0 ± 3.0	<0.001
Serum Fe	$\mu\text{g}/\text{dl}$	48	120.00 ± 6.0	61.0 ± 6.0	<0.001

Part II.

To evaluate the *in vivo* role of glucagon in mediating the metabolic response to septic stress a pancreatectomized rhesus monkey was developed. Results of experimental manipulation beyond pancreatectomy are preliminary. Nevertheless, glucagon appears to convey a significant part of the message signaling conversion of [^{14}C]-Ala to glucose.

Part III.

To test for increased glucagon and growth hormone and decreased insulin effect during amino acid vs. dextrose infusion, a study was conducted at WRAH

just prior to assignment of this investigator to USAMRIID. There 31 patients with normal pancreatic and hepatic function scheduled for similarly traumatic abdominal surgery were randomly assigned to receive postoperative maintenance infusions of 3.5% amino acid (fibrin hydrolysate) or dextrose 5% in 1/3 normal saline. The study which lasted 3 or 4 days ended when oral nutrition resumed. Blood was drawn at 1100 hours. Total urine collections were made daily and assayed for catabolites reflecting activity of mediator hormones. Several of the assays were performed at USAMRIID. Cortisol and catechol were similar in both groups as measured by 17-hydroxycorticosteroid, VMA and metanephrine excretion. In the amino acid group, however, ketoaciduria was always present indicating significant glucagon activity ($P < 0.001$). Serum glucagon was higher, significantly so on day 2 ($P < 0.05$). Growth hormone activity, as evidenced by OH-proline excretion, was increased (2143 ± 260 vs 494 ± 94 , $P < 0.001$). Serum insulin was decreased but never significantly. Urine 3-methylhistidine and nitrogen balance determinations corroborated protein sparing (both, $P < 0.05$). These data tend to support the new growth hormone-glucagon hypothesis.

Presentation:

Kaminski, M., N. Dunn, R. Wannemacher, R. Dinterman, R. DeShazo, W. Wilson, J. Earll and D. Carlson. Mechanisms for protein sparing during postoperative dextrose and free amino acid infusions. Presented, American Society for Clinical Nutrition, Atlantic City, NJ., 1 May 1976 (Clin. Res. 24:501A, 1976).

Publication:

Kaminski, M. V., H. A. Neufeld, R. W. Wannemacher, Jr., J. A. Pace, M. L. Armstrong, and R. E. Dinterman. Starvation and nonseptic trauma vs. sepsis and sepsis plus trauma - some specific metabolic differences. Abstr. Surg. Forum, 1976. In press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY						I. AGENCY ACCESSION ²		II. DATE OF SUMMARY ³		REPORT CONTROL SYMBOL					
3. DATE PREV SURVEY		4. KIND OF SUMMARY		5. SUMMARY SEC ⁴		6. WORK SECURITY ⁵		7. REGRADING ⁶		8. DISSEM INSTRN ⁷		9. SPECIFIC DATA - CONTRACTOR ACCESS		10. LEVEL OF SUM	
76 02 11		D. CHANGE		U		U		NA		NL		<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO		A. WORK UNIT	
10. NO./CODES: ⁸		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER				WORK UNIT NUMBER					
B. PRIMARY		61101A		3A161101A91C		00								138	
B. CONTRIBUTING														034/02/111	
C. CARRIED BY		CARDS 114(e)(f)													
11. TITLE (Provide with Security Classification Code) (U) Efficacy of zinc treatment in experimental endotoxemia and bacterial sepsis															
12. SCIENTIFIC AND TECHNOLOGICAL AREAS 003500 Clinical medicine; 004900 Defense; 012300 Biochemistry															
13. START DATE 76 02			14. ESTIMATED COMPLETION DATE CONT			15. FUNDING AGENCY DA			16. PERFORMANCE METHOD C. In-house						
17. CONTRACT/GRANT									18. RESOURCES ESTIMATE			19. PROFESSIONAL MAN YRS			
B. DATES/EFFECTIVE:			EXPIRATION:						FISCAL	PREVIOUS	0.1	20. FUNDS (in thousands)			
D. NUMBER: ⁹ NA									YEAR	CURRENT	5.0				
E. TYPE:			G. AMOUNT:							77	1.0	20.0			
F. KIND OF AWARD:			F. CUM. AMT.												
20. RESPONSIBLE DOO ORGANIZATION						21. PERFORMING ORGANIZATION									
NAME: ¹⁰ USA Medical Research Institute of Infectious Diseases ADDRESS: ¹¹ Fort Detrick, MD 21701						NAME: ¹⁰ Physical Sciences Division USAMRIID ADDRESS: ¹¹ Fort Detrick, MD 21701			PRINCIPAL INVESTIGATOR (Provide DOAN if U.S. Academic institution) NAME: ¹² Sobocinski, P. Z. TELEPHONE: 301 663-7181 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: Powanda, M. C. NAME: Canterbury, W. J.			POC:DA			
22. KEYWORDS (Provide each with Security Classification Code) (U) Zinc; (U) Prophylaxis; (U) Therapy; (U) Endotoxemia; (U) Gram-negative sepsis; (U) Military medicine; (U) BW defense; (U) Zinc															
23. TECHNICAL OBJECTIVE, ¹³ 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Proceed next of each with Security Classification Code.)															
23 (U) Determine and characterize prophylactic and therapeutic efficacy of zinc treatment and mechanisms involved. Provide information useful in establishing prophylactic and/or therapeutic regimen for endotoxemia arising either directly or indirectly from exposure to potential BW agents, or bacterial sepsis of importance to military medicine.															
24 (U) Septic or endotoxemic rats will be treated with zinc. Dosage timing and efficacy will be evaluated. A variety of biochemical changes will be monitored in order to determine mechanisms involved.															
25 (U) 76 02 - 76 06 - Pretreatment of rats with intraperitoneal injection of zinc chloride (1.6 mg/100 gm body weight) either one hour prior to, or simultaneous with, the IP administration of a lethal dose of endotoxin (1.0 mg/100 gm body wt of either <i>Salmonella typhimurium</i> or <i>Escherichia coli</i>) significantly enhances survival. In addition to enhanced survival endotoxin-induced hyperaminoacidemia and increased plasma lysosomal hydrolase activity were abated in zinc-pretreated rats. The protective effect of zinc appears to be related, in part, to its well documented role in the stabilization of biological membranes and the prevention of endotoxin-induced hepatocellular damage.															
Publications: Fed. Proc. 35:360, 1976. Clin. Chem. 22:1394-1396. 1976.															
Warning to contractors upon distributor's behalf.															
DD FORM 1498 1 MAR 68 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE. * U.S. GPO: 1974-840-843/8691															

BODY OF REPORT

Project No. 3A161101A91C: In-House Laboratory Independent Research (U)
(3A762760A834):

Task No. 3A161101A91C 00:
(3A762760A834 02): (Prevention and Treatment of Biological Agent Casualties)

Work Unit No. 91C 00 138: Efficacy of Zinc Treatment in Experimental
(834 02 111): Endotoxemia and Bacterial Sepsis

Background:

Experimentally-induced endotoxemia or that which accompanies gram negative sepsis often terminates in death of the animal.¹ Although numerous compounds provide varying degrees of protection against endotoxin lethality, e.g., heparin, cortisol, and others,² no concerted effort has been made to evaluate the potential protective effect of Zn. It is well known that endotoxin labilizes lysosomal membranes³ and that Zn stabilizes biological membranes.⁴ Zn is of further interest since endotoxin and sepsis are known to alter its tissue distribution. The present study is designed to determine the efficacy of Zn prophylaxis and therapy of endotoxemia. Several hypotheses concerning the mechanism(s) through which this trace metal might provide protection include the following: (1) inhibition of release of potentially harmful lysosomal hydrolases could promote survival by preventing tissue necrosis and/or activation of toxic peptide fragments such as bradykinin and myocardial depressant factor; and (2) influence endotoxin-induced release of clotting factors from granulocytes or enhance endotoxin detoxification by these cells since granulocytes appear normally to require and sequester relatively large amounts of Zn.

Progress:

Studies were performed to determine and characterize the efficacy of Zn treatment in experimental endotoxemia in rats and to obtain information on the mechanism(s) involved in its protective effect. Results indicate that either pretreatment or simultaneous administration of Zn, administered IP as ZnCl₂ (1.6 mg/100 gm body wt) significantly enhances survival of rats challenged² 1 hr later with an LD₈₀ of either Salmonella typhimurium or Escherichia coli lipopolysaccharide. Furthermore, the dose-response data indicate that lower doses (down to approximately 0.4 mg/100 gm body wt) of Zn are also effective. Its effectiveness is dependent on the time of treatment relative to the endotoxin challenge. The protection effect is essentially absent if Zn is administered from 1-5 hr after endotoxin.

Since Zn reportedly stabilizes and endotoxin labilizes biological membranes, measurements were made of plasma levels of several intracellular enzymes (β -glucuronidase, glutamic pyruvic transaminase and ornithine carbamyltransferase). Results indicate that Zn pretreatment prevents endotoxin-induced elevations in all of these enzymes. Since elevations in plasma ornithine carbamyltransferase levels are primarily associated with liver pathology, histological studies were performed. Results indicate that Zn pretreatment also prevents hepatocellular damage induced by endotoxin administration. Whether prevention of hepatocellular damage itself is sufficient to promote survival in treated rats is unknown and further studies are required.

Because of the importance of the liver in maintaining plasma amino acid homeostasis and the observed hepatocellular effect of endotoxin, studies were performed to determine whether (1) endotoxin alters plasma amino acid levels and (2) Zn pretreatment influences these alterations. Results obtained indicate that pretreatment prevents endotoxin-induced hyperaminoacidemia. Based on the available evidence, it appears unequivocal that at least part of the protective effect of Zn treatment in endotoxemia is attributable to prevention of hepatocellular damage.

Presentations:

Sobocinski, P. Z., M. C. Powanda, and W. J. Canterbury. Effect of zinc pretreatment on endotoxin-induced mortality and hyperaminoacidemia in rats. Presented, Annual Meeting, FASEB, Anaheim, CA, 11-16 April 1976. (Fed. Proc. 35:360, 1976).

Publication:

Sobocinski, P. Z., W. J. Canterbury and K. H. Jurgens. Improved continuous-flow method for determination of total serum hexoamines. Clin. Chem. 22: in press.

LITERATURE CITED

1. Nagler, A. L., and S. M. Levenson. 1971. Experimental hemorrhagic and endotoxic shock. p. 341-398. In *Microbial Toxins*, Vol. V (S. Kadis, G. Weinbaum, and S. J. Ajl, eds.) Academic Press, New York.
2. Berry, L. J. 1971. Metabolic effects of bacterial endotoxins. p. 165-208. In *Microbial Toxins*, Vol. V (S. Kadis, G. Weinbaum and S. J. Ajl, eds.) Academic Press, New York.
3. Weissmann, G., and L. Thomas. 1962. Studies on lysosomes. I. The effects of endotoxin, endotoxin tolerance, and cortisone on the release of acid hydrolases from a granular fraction of rabbit liver. J. Exp. Med. 116:433-450.
4. Chvapil, M. 1973. New aspects in the biological role of zinc: a stabilizer of macromolecules and biological membranes. Life Sci. 13:1041-1049.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ¹	2. DATE OF SUMMARY ²	REPORT CONTROL SYMBOL
3. DATE PREV SURVEY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCRT ³ U	6. WORK SECURITY ⁴ U	DA 0B6419	76 07 01	DD-DR&E(AR)636
10. NO./CODES: a. PRIMARY 62760A	PROGRAM ELEMENT	PROJECT NUMBER 3A762760A834	7. REGRADING ⁵ NA	8. DISSEMIN INSTN'H NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM a. WORK UNIT
b. CONTRIBUTING	c. ADDITIONAL CARDS 114(e)(f)		02		300	
11. TITLE (Prestack with Security Classification Code) (U) Immunologic studies with tick-borne rickettsiae						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁶ 003500 Clinical medicine; 004900 Defense; 010100 Microbiology						
13. START DATE 71 06	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house			
17. CONTRACT/GRANT	18. RESOURCES ESTIMATE PRECEDING FISCAL YEAR	19. PROFESSIONAL MAN YRS 1.0	20. PERFORMING ORGANIZATION NAME: Rickettsiology Division USAMRIID ADDRESS: Fort Detrick, MD 21701			
a. DATES/EFFECTIVE: b. NUMBER: NA	EXPIRATION:	21. CUM. AMT. 77	22. SOCIAL SECURITY ACCOUNT NUMBER: NAME: Kenyon, R. H. TELEPHONE: 301 663-7465 ASSOCIATE INVESTIGATORS NAME: Williams, R. G.			
c. TYPE:	d. AMOUNT:	e. CUM. AMT.	POC:DA			
g. KIND OF AWARD:						
19. RESPONSIBLE DOD ORGANIZATION NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		20. PERFORMING ORGANIZATION NAME: Rickettsiology Division USAMRIID ADDRESS: Fort Detrick, MD 21701				
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		21. GENERAL USE Foreign intelligence considered				
22. KEY WORDS (Prestack with Security Classification Code) (U) Immunology; (U) Vaccines; (U) Tick-borne rickettsiae; (U) Laboratory animals; (U) Human volunteers; (U) Military medicine; (U) BW Defense						
23. TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Prestack individual paragraphs identified by number. Prestack text of each with Security Classification Code.) 23 (U) Develop vaccines of low reactogenicity for immunoprophylaxis against specific rickettsial diseases. This work unit is part of a comprehensive program for developing new medical vaccines for defense against potentially important spotted fever rickettsial BW agents. 24 (U) Propagate representative strains in tissue culture systems. Assess the feasibility of producing rickettsial suspensions of quality and quantity suitable for vaccines for human use. 25 (U) 75 07 - 76 06 - The chick embryo cell-grown formalin-killed R. rickettsii vaccine has been approved by the Army Investigational Drug Review Board. Phase I of human testing has begun. Two prototype vaccines protecting against the tick-borne rickettsioses of the Eastern and Western hemisphere were prepared and tested for efficacy in guinea pigs. One of these vaccines (a mixture of Rickettsia rickettsii and Rickettsia conori) demonstrated protection against the most militarily significant tick-borne rickettsii. Cell-mediated immunity against tick-borne rickettsii was measured in humans by a lymphocyte transformation (LT) test and in guinea pigs by a macrophage inhibition factor (MIF) test as well as the LT test. LT was demonstrated in humans convalescent from infection with R. rickettsii and 3 of 4 individuals with heavy exposure to R. rickettsii but who had no symptoms of disease. In guinea pigs, both MIF and LT appeared between 1 and 2 weeks and was maintained for at least 6 weeks after infection. MIF activity was, but LT was not, demonstrated in vaccinated guinea pigs. Publications: J. Clin. Microbiol. 1:500-503, 2:300-304, 1975; 3:513-518, 1976 Infect. Immunity 12:1457-1463, 1975. Fed. Proc. 35:227, 1976						

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PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

* U.S. GPO: 1974-840-843/8691

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 300: Immunologic Studies with Tick-Borne Rickettsiae

Background:

Included among the tick-borne rickettsiae are R. rickettsii (the etiologic agent of Rocky Mountain spotted fever [RMSF]), the tick-borne rickettsioses of the eastern hemisphere, and rickettsialpox.¹ RMSF, found in the United States, is the most severe of these diseases. Rickettsia conorii, the etiological agent of fievre boutonneuse, South African tick bite fever and Indian and Kenyan tick typhus, causes a severe nonfatal infection, and is prevalent throughout parts of Europe, Africa and the Mediterranean basin. North Asian tick-borne typhus of Russia and Mongolia (Rickettsia siberica) also causes a severe nonfatal infection. Queensland tick typhus (Rickettsia australis) is a relatively benign mite-borne infection found in the United States and Russia. Due to the possibility of potential military traffic in selected geographic areas, Rickettsia rickettsii, R. conorii and R. siberica should be considered of military concern. Neither R. australis, due to its geographical isolation, nor R. akari, due to low virulence and incidence, is of military significance.

The only commercial tick-borne rickettsial vaccine currently available is the Cox-type R. rickettsii vaccine prepared from rickettsiae grown in chick egg yolk sacs and killed with formaldehyde.² Dupont et al.³ studied this vaccine in prisoners and concluded it provided little or no immunity. Our purpose was to produce an improved vaccine which offered substantial protection against R. rickettsii and to study the feasibility of producing a vaccine to protect against all tick-borne rickettsiae producing disease in man.

Measurement of immunity to tick-borne rickettsiae continues to be an enigma. Although several methods of antibody measurement are available, their correlation with protection is disputable. Cell-mediated immunity may be a more accurate measurement of immune status.

Progress:

PART I - Vaccine Studies

The request to the Army Investigational Drug Review Board for human immunization trials on our chick embryo cell (CEC) R. rickettsii vaccine was approved. Phase I safety testing of this vaccine in 6 human subjects has been initiated. Results at the present stage can be found in Work Unit 834 02 417.

Current vaccine study is concentrated on the feasibility of a multivalent product protecting against all or at least the most important diseases of the tick-borne rickettsiae. A prototype vaccine was prepared from R. rickettsii and R. australis. Guinea pigs were immunized with 3 injections 1 wk apart (each injection contained 3.1×10^8 R. rickettsii and 5.0×10^8 R. australis) and were challenged with one of the 5 tick-borne rickettsiae 30 days after the last inoculation. Mice, which are susceptible to R. akari, were injected by the same schedule but with half the volume used for guinea pigs (Table I).

TABLE I. REACTIONS AFTER CHALLENGE OF GUINEA PIGS AND MICE VACCINATED WITH R. RICKETTSII-R. AUSTRALIS VACCINE

CHALLENGE RICKETTSIAE	RICKETTSEMIA	NO./TOTAL	SCROTAL REACTION (0 to +4)	AVERAGE NO. GUINEA PIG DEGREE DAYS
<u>R. australis</u>				
vaccinated	-	0/6	+1	0.6
nonvaccinated	-	0/6	+2	3.0
<u>R. rickettsii</u>				
vaccinated	-	0/6	0	0
nonvaccinated	+	4/6	+4	6.0
<u>R. siberica</u>				
vaccinated	-	0/6	+1	0.9
nonvaccinated	+	0/6	+3	2.4
<u>R. conorii</u>				
vaccinated	-	0/6	+1	0.3
nonvaccinated	-	0/6	+3	1.2
<u>R. akari</u>				
vaccinated	-	0/5	+3	1.8
nonvaccinated	-	0/6	+4	4.1
<u>R. akari</u> (mice)				
vaccinated	+	0/20		
nonvaccinated	+	15/20		

Other than protection against the 2 homologous strains, the mouse death data indicated that this vaccine conferred protection against R. akari infection. Rickettsemia data suggested some protection against R. siberica; however,

little information could be inferred concerning protection against R. conorii since it causes such a mild disease in unprotected guinea pigs.

A second prototype vaccine was prepared from R. rickettsii and R. conorii. Guinea pigs vaccinated with two 1-ml IP inoculations ($\sim 10^8$ each/ml) spaced 2 wk apart were challenged 1 mon after the last inoculation. Mice were vaccinated with one-half that used for guinea pigs. Death and fever data after challenge are shown in Table II.

TABLE II. PROTECTIVE EFFICACY IN GUINEA PIGS AND MICE OF
R. RICKETTSII-R. CONORII COMBINED VACCINE

CHALLENGE RICKETTSIAE	VACCINATED			NONVACCINATED		
	Average No. Guinea Pig Degree Days ^a	Deaths/Total		Average No. Guinea Pig Degree Days	Deaths/Total	
<u>R. australis</u>	1.6	0/4		2.0	1/4	
<u>R. conorii</u>	0	0/6		1.0	1/4	
<u>R. siberica</u>	0	0/6		3.1	0/4	
<u>R. rickettsii</u>	0.1	0/6		5.5	3/6	
<u>R. akari</u> (mice)		7/10			10/10	

^aAverage number of degree days = sum of number of degrees > 103.8 F in any one group/total number of guinea pigs in that group.

There appeared to be good protection against R. rickettsii, R. siberica and, as nearly as can be determined using this model, against R. conorii. There appeared to be borderline protection against R. akari and R. australis, neither of which is of great military significance. From a military point of view, it seems that the combination of R. rickettsii and R. conorii would provide the most probable needed protection against tick-borne rickettsiae. Further studies on the efficacy of the vaccine are underway.

PART II - Immunological Studies

A study of the comparison of available serological methods to detect antibody against R. rickettsii was completed. These include CF, Weil-Felix, microagglutination (MA), fluorescent antibody (FA), and a radiometabolic technique. These studies on the sequence of humoral antibody events initiated

by R. rickettsii infection indicate that the FA and MA tests are the most sensitive, and antibody can be first detected by these methods at about the time of maximum rickettsemia.

However, these serological techniques often yield inconsistent results. Cell-mediated immunity (CMI) may be a more accurate assessment of the immune status. CMI was measured in humans and guinea pigs by the lymphocyte transformation (LT) test (in cooperation with Work Unit 834 02 417). In humans, little or no LT was found in naive individuals or in those vaccinated with commercial R. rickettsii vaccine. All individuals who had sustained an accidental laboratory R. rickettsii infection (N=4) showed a vigorous LT response. In addition, 3 of 4 individuals with heavy exposure to R. rickettsii also showed a vigorous LT response. There was no obvious correlation between LT and serological titers. Plans are to use this technique to monitor R. rickettsii laboratory infection and response to experimental R. rickettsii vaccines.

LT was followed in response to R. rickettsii infection of guinea pigs. Since R. rickettsii infection has about 50% mortality in untreated guinea pigs, the effect of rescue with tetracycline was examined (Table III).

TABLE III. EFFECT OF TETRACYCLINE ON LYMPHOCYTE STIMULATION INDICES OF GUINEA PIGS INFECTED WITH R. RICKETTSII

TREATMENT	STIMULATION INDICES ^a BY WEEKS					
	0	1	2	3	4	5
No tetracycline	2.1	1.0	13.2	10.0	18.0	16.2
Tetracycline ^b	1.2	4.8	4.9	10.8	21.6	29.1

^aRatio of counts/min in presence of antigen divided by counts/min without antigen.

^b20 mg/kg tetracycline on days 3 and 4 of fever.

In the nonrescued group, none of the animals showed a rise in LT at 1 wk; whereas in the rescued group, most animals showed some rise. LT in both groups rose in the following weeks. Magnitude of LT response did not seem to be affected by tetracycline rescue. Studies are presently underway to determine the effect, if any, of early vs. late antibiotic rescue.

No LT could be detected in guinea pigs vaccinated with 3 weekly injections of our CEC-grown R. rickettsii vaccine. Guinea pigs were then challenged with live R. rickettsii and one-half treated with tetracycline and the remainder not treated. Those treated with antibiotic failed to show any LT in the 3 wk after challenge, whereas the untreated guinea pigs showed LT as early as the first week after challenge (Table IV).

TABLE IV. EFFECT OF TETRACYCLINE ON LYMPHOCYTE STIMULATION INDICES AFTER CHALLENGE OF R. RICKETTSII-VACCINATED GUINEA PIGS

TREATMENT	STIMULATION INDICES ^a BY WEEKS			
	0	1	2	3
No tetracycline	2.1	5.5	22.0	23.5
Tetracycline ^b	2.2	1.8	2.0	1.6

^aRatio of counts/min in presence of antigen divided by counts/min without antigen.

^b20 mg/kg tetracycline on days 3 and 4 of fever.

As shown in Table III, LT does not appear in untreated guinea pigs until the second week. This suggests that prior exposure to the killed rickettsiae (vaccine) may prime the animals to elicit an earlier LT response to the antigen upon challenge. The fact that no LT response could be detected in the tetracycline-treated group suggests that after challenge of the vaccinees, an infectious process is initiated which is responsible for the LT response in the untreated group. This may be aborted by tetracycline therapy.

LT antigens for R. conorii and R. siberica have been prepared and significant LT (stimulation index ~10-15) in guinea pigs can be detected. Plans are to use these tests to study cross-reactions within the tick-borne rickettsiae.

In conjunction with the LT test, the direct macrophage inhibition factor (MIF) test was used to monitor CMI in guinea pigs (Table V). All guinea pigs were treated with antibiotic on days 3 and 4 of fever. MIF activity appeared as early as the first week after infection and was maintained through at least the fourth week. This is similar to the response found for the LT test. However, whereas no LT could be detected with vaccinated guinea pigs, a vigorous MIF response was detected.

TABLE V. PERCENT INHIBITION OF MACROPHAGE MIGRATION IN R. RICKETTSII
INFECTED OR VACCINATED GUINEA PIGS

GUINEA PIGS	% INHIBITION BY WEEKS AFTER INFECTION OR VACCINATION			
	1	2	3	4
Infected				
Trial 1	51	79	70	74
2	45	39	68	66
3	37	ND	ND	61
Normal				
Trial 1	7	-4 ^a	13	15
2	9	12	40	-16
3	-16	ND	ND	11
4		10		
5			12	
Vaccinated^b				
Trial 4			85	
5		84		

^aA negative number denotes enhancement of MIF activation.

^bTwo 0.5-ml immunizations (CEC-grown R. rickettsii vaccine) 2 wk apart.

Presentation:

Kenyon, R. H., and M. S. Ascher. Lymphocyte transformation (LT) to rickettsial antigen in Rocky Mountain spotted fever (RMSF). Presented, Annual Meeting, FASEB, Anaheim, CA, 12-16 April 1976. (Fed. Proc. 35:227, 1976).

Publications:

1. Kenyon, R. H., and C. E. Pedersen, Jr. 1975. Preparation of Rocky Mountain spotted fever vaccine suitable for human immunization. J. Clin. Microbiol. 1:500-503.
2. Kenyon, R. H., L. S. Sammons, and C. E. Pedersen, Jr. 1975. Comparison of three Rocky Mountain spotted fever vaccines. J. Clin. Microbiol. 2:300-304.

3. Buhles, W. C., D. L. Huxsoll, G. Ruch, R. H. Kenyon, and B. L. Elisberg. 1975. Evaluation of primary blood monocyte and bone marrow cell culture for the isolation of Rickettsia rickettsii. *Infect. Immun.* 12:1457-1463.

4. Kenyon, R. H., P. G. Canonico, L. S. Sammons, L. R. Bagley, and C. E. Pedersen, Jr. 1976. Antibody response to Rocky Mountain spotted fever. *J. Clin. Microbiol.* 3:513-518.

LITERATURE CITED

1. Woodward, T. E., and E. B. Jackson. 1965. Spotted fever rickettsiae. p. 1095-1129. In *Viral and Rickettsial Infections of Man* (ed. by F. L. Horsfall, Jr. and I. Tamm). J. Lippincott Co., Philadelphia, PA.
2. Cox, H. R. 1939. Rocky Mountain spotted fever. Protective value for guinea pigs of vaccine prepared from rickettsiae cultivated in embryonic chick tissues. *Pub. Health Rep.* 54:1070-1077.
3. DuPont, H. L., R. B. Hornick, A. T. Dawkins, G. G. Heiner, I. B. Fabrikant, C. L. Wisseman, Jr., and T. E. Woodward. 1973. Rocky Mountain spotted fever: comparative study of the active immunity induced by inactivated and viable pathogenic Rickettsia rickettsii. *J. Infect. Dis.* 128:340-344.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^b DA OD6421	2. DATE OF SUMMARY ^b 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SURVEY 76 02 23	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SECY ^a U	6. WORK SECURITY ^a U	7. REGRADING ^b NA	8. DRSIN' INSTN'R ^b NL	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: ^a PROGRAM ELEMENT 61101A	PROJECT NUMBER 3A161101A91C	TASK AREA NUMBER 00		11. LEVEL OF SUM A. WORK UNIT 134		
11. TITLE (Proceed with Security Classification Code) (U) Physicochemical and biological characterization of components of <i>Coxiella burnetii</i>						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry						
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27. KIND OF AWARD:		NAME: Rickettsiology Division USAMRIID ADDRESS: Fort Detrick, MD 21701		NAME: Wachter, R. F.		
28. RESPONSIBLE DOO ORGANIZATION		29. TELEPHONE: 301 663-7465		TELEPHONE: 301 663-7465		
30. RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		31. SOCIAL SECURITY ACCOUNT NUMBER:		SOCIAL SECURITY ACCOUNT NUMBER:		
32. GENERAL USE Foreign intelligence considered		33. ASSOCIATE INVESTIGATORS NAME: NAME:		34. POC:DA		
35. KEY WORDS (Proceed EACH with Security Classification Code) (U) Vaccines; (U) Q fever; (U) Rickettsia; (U) Military medicine; (U) BW defense						
36. TECHNICAL OBJECTIVE, ^b 36. APPROACH, 36. PROGRESS (Provide individual paragraphs identified by number. Proceed rest of each with Security Classification Code.) 23 (U) Demonstrate vaccine potential of components of <i>C. burnetii</i> to protect troops against Q fever from natural contact or from potential employment as a biological warfare agent.						
24 (U) Isolate purified components of <i>C. burnetii</i> ; determine antigenic, immunogenic, allergenic and physicochemical properties.						
25 (U) 75 07 - 76 06 - One injection (12 micrograms protein) of the soluble phase I antigen of <i>C. burnetii</i> completely protected guinea pigs against 10,000 median infectious doses of <i>C. burnetii</i> and 0.12 microgram provided partial protection, even though no phase I complement fixation antibody was detectable prior to challenge. Moreover, 3 doses of the soluble antigen failed to elicit any phase I complement fixation antibody; however, 14 days after challenge 100% of the immunized guinea pigs possessed this antibody in contrast to zero titer for all challenged nonimmunized animals.						
A component present in trichloroacetic extracts of phase I <i>C. burnetii</i> , which could be separated from other components by paper or thin layer chromatography, was not present in chromatograms of extracts of phase II <i>C. burnetii</i> . This technique may permit characterization studies of the phase I antigen in a state of purity not heretofore available.						
Publications: Infect. Immunity 12:433-436, 1975. Acta Virol. 19:500, 1975.						

^a Available to contractors upon originator's approval.

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1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

* U.S. GPO: 1974-840-843/8891

BODY OF REPORT

**Project No. 3A061101A91C: In-House Laboratory Independent Research
(3A762760A834)**

**Task No. 3A061101A91C 00:
(3A762760A834 02) Prevention and Treatment of Biological Agent Casualties**

Work Unit No. 91C 00 134: Physicochemical and Biological Characterization of Components of Coxiella burnetii

Background:

Last year we described the concentration of the soluble phase I antigen of C. burnetii by sulfite precipitation and results of a test to compare the immunogenicity of the resultant sulfite-antigen complex with the soluble antigen itself.¹ Briefly, one injection of the soluble antigen (30 µg protein) or of the sulfite complex (12 µg protein), in either soluble or particulate form, completely protected guinea pigs against 10⁴ ID₅₀ of phase I C. burnetii although no CF or microagglutinating (MA) antibodies were detectable prior to challenge. Most control animals failed to develop any antibody within 15 days after challenge; 90% of immunized animals possessed phase I and II MA and phase II CF antibodies at this time, but only 8% had phase I CF antibody.

A second immunogenicity test has been conducted to investigate further the patterns of antibody development produced by the phase I antigen. Also, preparatory to a comprehensive test to compare the efficacy of the Q fever vaccine in current use at USAMRIID with the Merrell-National Laboratories Q fever vaccine (NDBR-105) and the phase I antigen, a preliminary experiment was initiated to establish what level of soluble antigen to use in the full scale test. Information on the physicochemical characterization of the phase I antigen is included.

Progress:

Patterns of antibody development elicited by soluble phase I antigen of C. burnetii. Guinea pigs were given 2 SC injections of phase I antigen (20 µg protein/injection) or 2 injections of the sulfite-antigen complex (8 µg/injection) in soluble form in EDTA or in particulate form in buffer. Animals were bled 2, 6, and 13 wk after immunization. No significant differences were found in average geometric mean titers of groups receiving the soluble antigen or the antigen complex. To illustrate the changes in antibody levels, the overall antibody response of the combined treated groups in terms of the percentage of animals positive is presented in Table I.

TABLE I. ANTIBODY RESPONSE OF GUINEA PIGS TO IMMUNIZATION WITH THE PHASE I ANTIGEN OF COXIELLA BURNETII

TYPE OF ANTIBODY	% OF ANIMALS POSITIVE BY WEEKS				
	Postvaccine 2	6	13	Postbooster 2	Postchallenge 2
CF-phase I	0	0	0	0	100
phase II	100	100	25	85	100
MA-phase I	33	13	0	85	75
phase II	100	85	0	85	85

At 2 wk postvaccination all serum samples were positive for phase II CF and MA antibodies and 33% were positive for phase I agglutinating antibody. At 6 wk, although titers were lower, 100% of the sera were still positive for phase II CF antibody and 85% for phase II MA antibody; only a few samples showed any phase I agglutinins. At 13 wk no MA antibodies were detected and only 25% of the guinea pigs possessed phase II CF antibodies. We had thought that phase I CF antibody (the so-called "late" antibody) might be present at the 6- or 13-wk interval. On the contrary, even though a phase I antigen had been employed, phase I CF antibody was conspicuously absent. At 17 wk, groups of animals were given booster doses of the respective vaccine preparations and bled 2 wk later. As indicated in Table I, booster doses produced a reappearance of phase II CF and MA antibodies and phase I MA antibody in 85% of the test animals, but still no phase I CF antibody was detected. Three days after the postbooster bleeding, guinea pigs were challenged IP with 10^4 ID₅₀ of phase I Henzerling strain C. burnetii. Temperatures were taken daily for 2 wk; based on temperature response, all treated animals were protected. Sera were collected 2 wk postchallenge. The CF I antibody response of treated and control animals was completely different: 100% of the treated animals possessed CF I antibodies (titer range 1:8-1:128), while controls had none. Phase II CF antibody was also present in 100% of the vaccinated animals (titer range 1:64-1:1024), and phase I and II agglutinating antibodies were present in 75% and 85% of these sera, respectively (Table I). Although administration of the phase I antigen failed to evoke phase I CF antibody response, guinea pigs that had received this antigen, when challenged, were apparently "primed" to produce rapidly the CF I antibody in contrast to the complete absence of this antibody in challenged control animals.

Preliminary experiment to compare immunogenicity of the soluble antigen of C. burnetii and the whole organism vaccine. A full scale test will be conducted in Rickettsiology Division, with Dr. James Johnson as the principal investigator (Work Unit 834 02 304), to compare the Merrell-National Q fever vaccine (NDBR-105)

with the Q fever vaccine in current use at USAMRIID (prepared at WRAIR in 1964 and designated "DP-7"), and with the soluble phase I antigen of C. burnetii. A preliminary experiment has been carried out to establish the level of soluble antigen for use in this test. Two preparations of soluble antigen were compared with the NDBR-105 vaccine: (1) a trichloracetic (TCA) extract and (2) a TCA extract of a concentrated rickettsial sediment prepared by Major Carl Pedersen from an outdated liquid Q fever vaccine (WRAIR, designated FP2315). Preparations were compared on the basis of protein content at levels of 5 and 1 μ g. The lyophilized Merrell-National vaccine, reconstituted to 13 ml, with a protein content of 330 μ g/ml, was diluted to the 5 μ g level. The TCA extract of the Merrell-National vaccine (dialyzed and filtered) had a protein content of 6.0 μ g/ml and was diluted to 5 μ g/ml. The TCA extract of the concentrate of vaccine FP2315, with a protein content of 28 μ g/ml, was diluted to 1.0 μ g/ml and tested only at that level. Guinea pigs, in groups of 4, were given 2 SC injections of the above preparations, with a 14-day interval between doses. Two weeks later, challenge was accomplished with 10^5 ID₅₀ of phase I Henzerling strain C. burnetii. Temperatures were recorded for 10 days; 104 C for > 2 consecutive days was considered indicative of lack of protection. All of the animals that received 5 or 1 μ g doses of the Merrell-National vaccine or 5 μ g doses of the TCA extract of this vaccine were protected. One guinea pig in the 1 μ g TCA-Merrell-National vaccine group and one in the 1 μ g TCA-vaccine FP2315 groups were unprotected, with elevated temperatures for 5 and 2 days, respectively. All control animals reacted positively with elevated temperatures for a total of 11 days. Serological data on pre- and postchallenge serum samples is not yet available, but challenge results indicate the approximate level of soluble phase I antigen that should protect 50% of the test animals.

Physicochemical characterization of C. burnetii. Investigations using thin layer and paper chromatography have been initiated with TCA extracts of the phase I antigen with the objective of separating the active antigenic component from other substances present in the extracts. Water caused all detectable substances to move with the front on paper and thin layer plates. However, with the isopropyl alcohol-ammonia-water (9:1:2) system, in addition to material at the solvent front, a spot with an R_f of 0.22 was present, as visualized by iodine vapor. When a TCA extract of a phase II preparation of C. burnetii was examined in the same system, material was seen only at the solvent front. Attempts will be made to remove substances from the cellulose or paper and test them for antigenicity and reactogenicity.

Presentation:

Wachter, R. F. Changes in buoyant density relationships of two cell types of Coxiella burnetii phase I. Presented, Symposium "Basic Biology of the Rickettsiae", WRAIR, Washington, D. C., 23-24 Oct 75.

Publications:

1. Wachter, R. F., G. P. Briggs, J. D. Gangemi, and C. E. Pedersen, Jr. 1975. Changes in buoyant density relationships of two cell types of Coxiella burnetii phase I. Infect. Immun. 12:433-436.

2. Wachter, R. F., G. P. Briggs, and C. E. Pedersen, Jr. 1975.
Precipitation of phase I antigen of Coxiella burnetii by sodium sulfite.
Acta Virol. 19:500.

LITERATURE CITED

1. Wachter, R. F., G. P. Briggs, and C. E. Pedersen, Jr. 1975.
Precipitation of phase I antigen of Coxiella burnetii by sodium sulfite.
Acta Virol. 19:500.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA OF6414	2. DATE OF SUMMARY 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUMRY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCRTY U	6. WORK SECURITY U	7. REGRADING NA	8. DISPN INSTN'R NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: a. PRIMARY 62760A	PROGRAM ELEMENT PROJECT NUMBER 3A762760A834			TASK AREA NUMBER 02	11. LEVEL OF SUM A. WORK UNIT WORK UNIT NUMBER 303	
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12. TITLE (Pecode with Security Classification Code) (U) Immunopathogenesis of tick-borne rickettsial diseases						
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RESPONSIBLE INDIVIDUAL		NAME: Metzger, J. F. TELEPHONE: 301 663-2833		NAME: Rickettsiology Division USAMRIID ADDRESS: Fort Detrick, MD 21701		
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24. ETCODES (Pecode each with Security Classification Code)		(U) BW defense; (U) Military medicine; (U) Antigen; (U) Early diagnosis: (U) Radioimmunoassay (RIA); (U) Laboratory animals				
25. TECHNICAL OBJECTIVE, 26. APPROACH, 28. PROGRESS (Pecode individual paragraphs identified by number. Pecode last of each with Security Classification Code.)		<p>23 (U) Develop a sensitive radioimmunoassay for the early detection of rickettsial antigens as a defense against covert or overt use of biologicals by unfriendly forces. Use this assay for the detection of antibodies or antigen-antibody complexes as a definitive aid in the diagnosis of rickettsial infection.</p> <p>24 (U) Modify an indirect RIA technique for use with rickettsiae to test the time relationship during experimental infections in guinea pigs and rabbits.</p> <p>25 (U) 75 07 - 76 06 - Research is directed toward elucidation of the role of immune complexes and acute immune complex disease as a causal factor in the pathogenesis of the vasculitis associated with the tick-borne group of rickettsial diseases. The first step in this work consisted of developing a sensitive RIA for antirickettsial antibody. After initial unsuccessful attempts with a liquid phase RIA system, a solid state system using rickettsial antigen bound to microtiter plate wells was developed. Comparison of an indirect assay (using labeled antibody specific for the particular rickettsia) and an indirect assay (using labeled antibody specific for globulins of the particular species of test animal) demonstrated the superior sensitivity and versatility of the latter. This indirect RIA, developed using monkey sera, was then modified for use with human sera. Finally, extensive modification is being made to increase the daily sample capacity, allowing for large scale serological studies. Additional studies have been initiated to develop an RIA to detect rickettsial antigens, and to investigate cell-mediated immunity to both rickettsial and unrelated antigens during experimental rickettsial diseases.</p>				

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1 MAR 68PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68
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BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 303: Immunopathogenesis of Tick-Borne Rickettsial Diseases

Background:

There have been no major developments in diagnosis or treatment of tick-borne rickettsiosis in 25 years. The case fatality rate has remained in the range 7-10%, and the incidence seems to be significantly increasing.

Of the many reasons advanced for the continued high case fatality rate, two seem plausible: (1) delayed or missed diagnosis, leading to inadequate treatment, and (2) occasional induction of acute immune-complex disease with fulminant arteritis, which progresses despite antimicrobial therapy.

The problem of delayed diagnosis lies partially in the fact that there is no currently available test that can confirm the diagnosis quickly; most are serologic tests that require paired acute and convalescent sera. Therefore, a radioimmunoassay (RIA) to detect antibodies to Rickettsia rickettsii during the course of experimental infection has been developed. Modification of this RIA might permit detection of the antigen and soluble immune complexes, and, therefore, serve as a tool to investigate the pathogenesis of tick-borne rickettsial infections.

Progress:

Initial studies on the development of a radioimmunoassay use the following procedure: particulate R. rickettsii antigen (Ag), Sheila Smith strain, partially purified from CEC cultures by rate zonal centrifugation is incubated with test serum (Ab_1) in PBS. Species-specific second antibody (Ab_2), labeled with ^{125}I by the lactoperoxidase method, is then added and incubation continued. The particulate immune complex, Ag- Ab_1 - Ab_2 - ^{125}I , is pelleted by centrifugation and counted in a γ -counter. Results are computed as percentage of the total CPM added which are retained in the pellet. Studies using this procedure have been unsuccessful in detecting the presence of anti-R. rickettsii Ab in known microagglutin (MA) positive sera, despite manipulations in reagents, equipment and techniques.

Due to lack of success with the preliminary technique, other approaches were investigated. An attempt was made to grow R. rickettsii in DEC monolayers in 3-ml vials to use as solid-state Ag for the assay for Ab_1 . Unfortunately,

we were unable to propagate rickettsiae in these vials because it proved nearly impossible to prevent contamination with bacteria in the antibiotic-free medium needed for growth of rickettsiae.

Finally, a solid-state system was devised, utilizing R. rickettsii Ag fixed to the walls of the wells of flexible plastic microtiter plates. First, a direct test was developed: human serum with a high anti-R. rickettsii titer was fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation, and the γ -globulin fraction labeled with ^{125}I by the lactoperoxidase method. This labeled anti-R. rickettsii globulin was then incubated in microtiter wells precoated with Ag, washed, separated, and counted. Using data from the direct test, the amount of antigen added per well, and concentration of labeled anti-R. rickettsii, globulin were selected that gave maximum CPM bound. Using this system, an inhibition assay for R. rickettsii antibody in unknown test sera was developed. Serial dilutions of the test serum are first incubated in the antigen-coated microtiter wells. These are washed, incubated a second time with the preselected concentration of labeled anti-R. rickettsii, washed again, separated, and counted. Titers of sera using this inhibition assay are similar to those obtained by MA tests, but this procedure offered no advantages in ease of performance; therefore, no further studies are planned using the inhibition assay.

An indirect assay employing labeled species-specific antiglobulin was then developed.

Broadly, the RIA procedure involves: (1) coating microtiter plate wells with R. rickettsii or control (normal chick embryo cell culture) Ag; (2) fixing the Ag to the wells; (3) a first incubation with dilutions of test sera; (4) a first wash; (5) a second incubation with species-specific antiglobulins (γG , γM , γA) labeled with ^{125}I by the lactoperoxidase method; (6) a second wash; (7) separation of the microtiter plate wells; and (8) counting in γ -counter.

Results are calculated as binding ratio (BR) of the CPM bound to R. rickettsii Ag (minus background) divided by the CPM bound to control Ag (minus background).

Each step of the RIA, as listed above, was studied independently to maximize BR as follows:

1. Serial dilutions of Ag showed BR maximum at 1:2 - 1:8.
2. Ag-coated microtiter wells were fixed with diethylether, methanol, ethanol, acetone, or gluteraldehyde. Only diethylether improved BR over that of unfixed control.
3. Diluent for the first incubation improved BR if small amounts of protein were added; the best diluent proved to be EMEM with 10% calf serum, with a broad pH optimum range of 7.2 - 7.8. Time of first incubation required for maximal BR is in the range 2-10 hr at 35 C.

4. BR maximizes at a dilution of 1:160 - 1:320 of ^{125}I -labeled anti-globulin in EMEM and 10% calf serum, pH 7.8, with the addition of 0.1 M EDTA.

5. Detergent wash after the second incubation period improves binding ratio by removing nonspecifically bound label.

From these data a procedure has been adopted for this RIA which has improved maximal BR.

1. Add 50 μl of 1:2 - 1:8 dilutions in PBS, pH 7.1, of R. rickettsii and control Ag to microtiter wells (the dilution used is dependent on the specific Ag preparation).

2. Air dry at 35 C.

3. Fix with cold diethylether, 100 μl /well.

4. Store in dessicator at 4 C until needed.

5. Add test sera at appropriate dilutions in EMEM with 10% calf serum, pH 7.8, to R. rickettsii Ag and control Ag wells, 50 μl /well.

6. Incubate 2 hr at 35 C.

7. Wash in tap water 10x.

8. Add appropriate dilution of ^{125}I -labeled species-specific antiglobulin in EMEM with 10% calf serum, 0.1 M EDTA, pH 7.8, 50 μl /well.

9. Incubate 2 hr at 35 C.

10. Wash with 2% Isoclean, 3x, and tap water, 10x.

11. Separate microtiter wells.

12. Count.

13. Calculate results.

This RIA was developed initially for detection of Ab_1 from monkey sera to one member of the tick-borne rickettsiae (R. rickettsii). The assay was then modified for two purposes: (1) detection of Ab_1 in human sera, and (2) conversion to a simpler technical approach that will allow processing of much larger numbers of sera each day.

The RIA, as originally conceived, required serial 2-fold dilutions of each test serum. A titer was then established as the last dilution of serum which had a binding ratio (counts bound to rickettsial Ag divided by counts bound to control Ag) significantly > 1 . Due to the necessity of serially diluting each

test serum, a maximum of only 2-4 assays could be performed each day. Therefore, a new approach was demanded to handle larger numbers of sera.

The new approach involves an initial screening of test sera for Ab_1 . Duplicate determinations of CPM bound to rickettsial Ag are compared to counts bound by several known negative controls. Sera with CPM greater than the CPM + 3 SD bound to negative controls are positive while those with counts < CPM + 3 SD are negative. Initial results with this screening procedure revealed best discrimination between known positive and negative sera at a dilution of about 1:16 in PBS.

Titers are determined on the sera that are positive by this screening procedure. This is accomplished by plotting a standard curve with dilutions of a known positive serum. Two dilutions of test sera are then assayed and results are calculated from the standard curve. Results are now expressed as the dilution of the standard serum which gives the same binding as the test serum.

Additionally, monospecific IgG-antibody to R. rickettsii is being prepared for use in constructing the standard curve. Results will then be expressed as μ g of specific Ab_1 bound, rather than the titer obtained by dilution.

Attempts to convert the assay to use for human sera have been only partially successful. Restudy of each step in the procedure has identified several problems. Bacterial contamination of the Ag led to rapid deterioration of assay specificity; addition of sodium azide and storage of Ag at -70°C has eliminated this problem. Eliminating the use of control Ag necessitated the use of more purified Ag in order to prevent false-positive reactions; consequently, all rickettsial Ag is now purified by ultracentrifugation on sucrose gradients before use in the assay. Standardization of Ag preparations is achieved by dilution to 10^8 particles/ml, as determined by acridine orange stain.

Despite these refinements, the assay has little sensitivity in the human system. Tests have eliminated several possible explanations for this; however, two remain to be investigated: (1) radiation damage to the Ab_2 , destroying its affinity for human IgG, and (2) poor binding of Ag to the walls of the micro-titer plates. Studies will be performed employing a soluble Ag, prepared from the supernatant after sonication of R. rickettsii. Attachment using this small moiety should be more stable than the particulate whole organism Ag currently employed.

Studies are also underway to develop a RIA for R. rickettsii antigen. Rickettsia are being fractionated by sonication and the soluble antigen (see Work Unit No. 834 02 306) collected by ultracentrifugation. This portion will then be labeled with ^{125}I , and used in a competitive binding liquid phase RIA. Antibody for use in this system will be selected from sera collected from hyperimmunized rabbits.

Finally, a new phase of studies will be initiated correlating disappearance of Ag, appearance of Ab₁, and appearance of CMI as measured by skin testing and lymphocyte transformation during the course of experimental infection with R. rickettsii in guinea pigs.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION# DA OF6418	2. DATE OF SUMMARY# 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SURVY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SECY# U	6. WORK SECURITY# U	7. REGRADING# NA	8. DISPN INSTN# NL	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT
10. NO./CODES: a. PRIMARY 62 760A	PROGRAM ELEMENT PROJECT NUMBER 3A762760A834			TASK AREA NUMBER 02	WORK UNIT NUMBER 304		
b. CONTRIBUTING c. <i>for</i>	CARDS 114(e)(f)						
11. TITLE (Purcell with Security Classification Code) (U) Strain characteristics of the tick-borne rickettsiae							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS# 003500 Clinical medicine; 004900 Defense; 010100 Microbiology							
13. START DATE 75 03	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house				
17. CONTRACT/GRAANT		18. RESOURCES ESTIMATE					
a. DATES/EFFECTIVE:	EXPIRATION:	FISCAL	PROJECTIONS	a. PROFESSIONAL MAN YRS 76		b. FUNDS (in thousands) 146.3	
b. NUMBER: NA		YEAR	CURRENT	1.0		160.0	
c. TYPE:	d. AMOUNT:						
e. KIND OF AWARD:	f. CUM. AMT.						
19. RESPONSIBLE DOO ORGANIZATION							
NAME: USA Medical Research Institute of Infectious Diseases				NAME: Rickettsiology Division			
ADDRESS: Fort Detrick, MD 21701				ADDRESS: USAMRIID Fort Detrick, MD 21701			
20. RESPONSIBLE INDIVIDUAL							
NAME: Metzger, J. F. TELEPHONE: 301 663-2833				NAME: Johnson, J. W. TELEPHONE: 301 663-7465 SOCIAL SECURITY ACCOUNT NUMBER:			
21. GENERAL USE							
Foreign intelligence considered				ASSOCIATE INVESTIGATORS NAME: NAME: POC:DA			
22. KEYWORDS (Purcell EACH with Security Classification Code) (U) BW defense; (U) Military medicine; (U) Tick-borne rickettsioses; (U) Vaccines							
23. TECHNICAL OBJECTIVE, # 24. APPROACH, 25. PROGRESS (Purcell individual paragraphs identified by number. Purcell text of each with Security Classification Code.) (U) Characterize in vivo and in vitro biological markers of rickettsial cultures to facilitate differentiation among strains and species. Assess low virulence organisms of potential military importance for use as live vaccines for protection of troops exposed to a biological warfare environment.							
24. (U) Grow strains of tick-borne rickettsiae in embryonated eggs, tissue culture, and animals to select substrains with properties different from the parent, with special attention to virulence changes. Measure protective efficacy of low virulent strains to evaluate their potential as a live vaccine.							
25. (U) 75 07 - 76 06 - Stock cultures of high and low virulent strains of R. rickettsii were studied for virulence homogeneity by testing plaque isolates from tissue culture monolayers and from cultures partially inhibited by drugs. All plaques isolated were identical in virulence with the parent strain. Mixtures of the 2 strains could be separated by plaquing when in approximately equal proportions, but not when either strain was in 1000-fold excess. Passage in guinea pigs maintained the identity of the mixtures, but passage in embryonated eggs allowed the high virulent strain to overgrow the low virulent strain. A study of the tissue culture host range of 8 strains in cell lines has shown that plaque formation used as an indicator allowed strains to be distinguished fairly readily.							
A protocol was prepared for investigating the protective efficacy and other properties of a new phase I Q fever vaccine intended to replace the currently used vaccine.							
Publication: J. Infect. Dis. 133:334-338, 1976.							
Available to contractors upon ordinator's approval.							

DD FORM 1498
1 MAR 68PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

* U.S. GPO: 1974-540-843/8691

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 304: Strain Characteristics of the Tick-Borne Rickettsiae

Background:

The only reliable differentiating characteristic among strains of R. rickettsii so far reported has been virulence for guinea pigs. This virulence property was quantitated by Price¹ and appeared to be a reasonably stable marker, even though it varied temporarily under certain conditions. Other markers for strains of this organism such as growth characteristics, strain-specific antigens or biochemical reactions have proved unsuitable or have not been investigated. The present work unit was established to study the characteristics of strains of R. rickettsii to identify potential markers and, hopefully, to find some which might correlate with virulence. A second objective was to find and investigate strains of low virulence in an attempt to locate one or more which could serve as candidates for a live tick-borne rickettsial vaccine.

Progress:

The usual means by which R. rickettsii is propagated involves the introduction of a rather large inoculum of organisms into the growth system and the harvesting of progeny 4-7 days later. Because this procedure favors the development and maintenance of subpopulations of rickettsiae, the purity of the cultures was investigated first. Two strains used in these experiments, the low virulence Iowa and the high virulence Sheila Smith, were tested for homogeneity. Since virulence for guinea pigs was the most significant differentiating characteristic, it was chosen as the trait for testing. The 2 strains were grown in chick fibroblast, L and MK-2 cell lines; plaques were picked and grown out in embryonated eggs. These sublines and the parent strains were inoculated into guinea pigs to test for virulence by the method of Price; his designations are R, S, T, and U, from most to least virulent.¹ During these experiments, about 30 sublines were tested, 5 clones from each cell line for each rickettsial strain. In all cases, the virulence of the substrain matched that of the parent stock from which it was derived.

Sublines were also obtained from plaques of both rickettsial strains from cultures partially inhibited by the drugs p-aminobenzoic acid, p-hydroxybenzoic acid, salicylic acid and quinoxaline. These, or related compounds, were used by Weiss² to obtain drug-resistant strains of typhus rickettsiae for genetic studies,² and 2 strains, resistant to quinoxaline oxide and quinoxaline oxide-p-aminobenzoic acid, may have developed changes in their virulence properties.

However, the tick-borne rickettsiae substrains obtained from these drug-inhibited cultures were of the same virulence class as the parent strain.

Since these experiments were uniformly negative in detecting virulence differences among substrains of the parent stock strains, another approach was attempted. A series of mixtures of the 2 strains was prepared containing various levels of each, and attempts were made to recover the original strains. Mixtures were prepared having 1 egg LD₅₀ of Iowa (T) to 1000 of Sheila Smith (R), equal doses of each, and 1 egg LD₅₀ of Sheila Smith to 1000 of Iowa. Assayed in embryonated eggs, the mixtures showed 1.5×10^5 , 5.6×10^5 , and 1.4×10^5 LD₅₀/ml, and in guinea pigs, they reacted as R, R, and T strains, respectively. Attempts to reisolate the rickettsial strains were successful only in the equal dose mixture. With this mixture, random selection of plaques from all 3 cell lines yielded subcultures of both rickettsial strains. Little or no morphological variations in plaques were observed except perhaps in the L cell monolayers where Iowa plaques appeared slightly smaller and perhaps less well defined than Sheila Smith plaques.

The mixtures were then passaged serially in guinea pigs and embryonated eggs. All retained their original virulence state for 3 passages in guinea pigs, but in eggs the pattern changed. After 5 passages, yolk-sac suspensions of all mixtures were virulent for guinea pigs, the predominantly Iowa strain mixture had converted to an R type virulence level. Apparently, the Sheila Smith strain had overgrown the Iowa even with an initial 1:1000 disadvantage. If further studies indicate that more virulent strains overgrow the less virulent strains in eggs, this may provide a test procedure for detecting low levels of virulent organisms in a live vaccine, and may indicate another characteristic for measuring virulence.

Because of the limited number of markers available for distinguishing strains of *R. rickettsii*, other potential characteristics are being investigated. The most promising has been tissue culture host range as indicated by plaque formation. Seven strains of *R. rickettsii* were adsorbed on monolayers of 10 different tissue cell lines and plaque formation was observed. Table I summarizes the results of those combinations so far tested. These results were obtained under a standard set of conditions in which drained tissue culture monolayers were adsorbed with dilutions of the rickettsial strain for 1 hr at 34 C, overlayed with EMEM containing the nonessential amino acids and 0.5% agarose. Cultures were incubated at 34 C for 6 days, stained with neutral red and examined for plaque formation.

TABLE I. PLAQUE FORMATION BY TICK-BORNE RICKETTSIAE IN VARIOUS TISSUE CELL LINES

CELL LINE	PLAQUE FORMATION BY STRAINS						
	Sheila Smith	Bitter Root	Parkerii	Montana	Iowa	Bates	Maculatum
Chicken CF	+	+	+	+	+	+	+
Duck DF	+			+	+	+	
Monkey Vero	+	+	0	0	0	+	0
BS-C-1	+	+	+		0	+	0
MK-2	+	0	+/-		+	+	
Hamster BHK	+	+	0		0	0	
Mouse L	+	+	+	+	+	+	+
Human WI-38	+	0		0	+	0	0
WISH	+	0	+		+	+	+/-
HeLa	+/-			0	0	0	

It appears from data in Table I, that these rickettsial strains can be differentiated by plaque formation in appropriate tissue culture cell lines. In general, the differentiation among these strains did not seem to be related to virulence. However, plaque formation in one cell culture, the BHK line, did appear related; the more virulent strains produced plaques, while the less virulent did not. The Sheila Smith strain, the most virulent of the group, produced plaques in all cell lines tested, with the possible exception of HeLa cells. Here, areas in the cell sheet resembling plaques were occasionally seen, but well defined plaques were not found. Further work on host cell range of

rickettsial strains will continue and growth curves of the strains in various cell lines will be developed. These results along with virulence markers will be used in mixed strain studies to determine whether growth rate is a virulence factor.

A protocol has been developed for comparing the protective efficacy of the currently used Q fever vaccine with a formalin-killed vaccine prepared by the Merrell-National Laboratories under contract with USAMRIID (See Work Unit 834 02 301). For this study, guinea pigs will be vaccinated SC with single doses of various dilutions of each vaccine. Three weeks later, they will be bled to determine serological responses to the vaccine, and challenged with a fever-inducing dose of virulent *Coxiella burnetii*. The temperature response of each guinea pig will be measured; from these data the relative efficacy of each vaccine will be determined. Serological tests will include CF, MA, and FA and RIA procedures. Other experiments will be conducted to determine the effectiveness of using multiple doses of vaccine.

Publication:

Johnson, J. W., G. A. Eddy, and C. E. Pedersen, Jr. 1976. Biological properties of the M-44 strain of *Coxiella burneti*. J. Infect. Dis. 133:334-338.

LITERATURE CITED

1. Price, W. H. 1953. The epidemiology of Rocky Mountain spotted fever. I. The characterization of strain virulence of *Rickettsia rickettsii*. Am. J. Hyg. 58:248-268.
2. Weiss, E. 1960. Some aspects of variation in rickettsial virulence. Ann. N. Y. Acad. Sci. 88:1287-1297.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a DA OF6421	2. DATE OF SUMMARY ^b 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SURVEY 73 07 28	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SECY ^c U	6. WORK SECURITY ^c U	7. REGRADING ^d NA	8. DSBIN INSTN ^e NL	9. SPECIFIC DATA-CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT
10. NO./CODES: ^f a. PRIMARY 62760A		PROJECT NUMBER 3A762760A834		TASK AREA NUMBER 02	WORK UNIT NUMBER 305		
b. CONTRIBUTING							
c. EQUIPMENT CARDS 114(e)(f)							
11. TITLE (Proceed with Security Classification Code) (U) Cellular immune response in rickettsial infections							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^g 003500 Clinical medicine; 004900 Defense; 010100 Microbiology							
13. START DATE 75 07	14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house			
17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS		20. FUNDS (in thousands)	
a. DATES/EFFECTIVE: b. NUMBER: c. TYPE: d. KIND OF AWARD:		EXPIRATION: NA	FISCAL YEAR PRECEDING 76	CURRENT 77	1.0	118.8	
e. AMOUNT: f. CUM. AMT.				1.0	92.0		
21. RESPONSIBLE DOO ORGANIZATION		22. PERFORMING ORGANIZATION					
NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		NAME: Rickettsiology Division USAMRIID ADDRESS: Fort Detrick, MD 21701					
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Punish DSN if U.S. Academic Institution) NAME: Rudczynski, A. B. TELEPHONE: 301 663-7465 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME:					
23. GENERAL USE Foreign intelligence considered				POC:DA			
24. KEY WORDS (Proceed with Security Classification Code) (U) Immunology; (U) Rickettsial diseases; (U) Cell-mediated immunity (CMI); (U) Antigens; (U) Complement fixation (CF); (U) BW defense							
25. TECHNICAL OBJECTIVE, ^h 26. APPROACH, 28. PROGRESS (Punish individual paragraphs identified by number. Proceed with code with Security Classification Code.) 23 (U) Investigate the role of CMI in hosts infected with rickettsiae to determine how humoral antibody-producing antigens are important. This work unit is part of a comprehensive program to develop effective new vaccines for medical defense against rickettsial BW agents. 24 (U) Inoculate laboratory animals with rickettsiae. Measure humoral and cell-mediated antibody responses. 25 (U) 75 07 - 76 06 - The role of CMI in animal hosts infected with rickettsiae was studied. Cell-mediated responses in rickettsial infections have not been fully explored. The ultimate objective of developing a vaccine has made it necessary to examine CMI. Guinea pigs were inoculated ID, IP, SC, IM or intracardially with 1 ml of 20% yolk-sac suspension (30-40 median infecting doses) of Rickettsia tsutsugamushi, Karp strain, a dose sufficient to induce a febrile response but no deaths. Animals were bled via cardiac puncture weekly and CF and Weil-Felix titers were determined. Determination of peripheral blood lymphocyte stimulation was made at 30 days. All routes of inoculation were effective in producing cells that could be stimulated in vitro (10-100 X) in the presence of UV-inactivated tissue-culture-grown (monkey BS-C-1 cells) antigen. All inoculated guinea pigs were skin tested and were positive with maximal induration and erythema occurring between 24 and 48 hr. The appropriate controls showed little or no reaction to skin testing or to lymphocyte stimulation by antigen. None of the inoculated animals showed any significant cross-reaction in the Weil-Felix test with Proteus OXK antigen. CF titers varied over a wide range and did not correspond to the degree of lymphocyte stimulation seen. These results suggest that a cell-mediated immune response to scrub typhus infection can be elicited in guinea pigs. Publication: Fed. Proc. 35:227, 1976.							

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1 MAR 68PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

* U.S. GPO: 1974-540-843/8691

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 305: Cellular and Immune Response in Rickettsial Infections

Background and Progress:

The role of cell-mediated immunity (CMI) in animal hosts infected with rickettsiae was studied. Cell-mediated responses in rickettsial infections have not been fully explored. The ultimate objective of developing a vaccine has made it necessary to examine CMI. Guinea pigs were inoculated intra-dermally, intraperitoneally, subcutaneously, intramuscularly or intracardially with 1 ml of 20% yolk-sac suspension (30-40 median infecting doses) of Rickettsia tsutsugamushi, Karp strain, a dose sufficient to induce a febrile response but no deaths. Animals were bled via cardiac puncture weekly and CF and Weil-Felix titers were determined. Determination of peripheral blood lymphocyte stimulation was made at 30 days. All routes of inoculation were effective in producing cells that could be stimulated in vitro (10-100 X) in the presence of UV-inactivated tissue-culture-grown (monkey BS-C-1) antigen. All inoculated guinea pigs were skin tested and were positive with maximal induration and erythema occurring between 24 and 48 hr. The appropriate controls showed little or no reaction to skin testing or to lymphocyte stimulation by antigen. None of the inoculated animals showed any significant cross-reaction in the Weil-Felix test with Proteus OXX antigen. CF titers varied over a wide range and did not correspond to the degree of lymphocyte stimulation seen. These results suggest that a cell-mediated immune response to scrub typhus infection can be elicited in guinea pigs.

The principal investigator has left the Army. The work unit will remain open but inactive pending assignment of a new individual.

Publication:

Rudczynski, A. B. 1976. Demonstration of cell-mediated immunity in guinea pigs infected with scrub typhus. Fed. Proc. 35:227.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^b	2. DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL
3. DATE PREV SURRY 76 02 11	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY ^b U	6. WORK SECURITY ^b U	7. REGRADING ^b NA	8. DOD/FN INSTN ^b NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: ^b PROGRAM ELEMENT				PROJECT NUMBER 3A161101A91C	TASK AREA NUMBER 00	D. LEVEL OF SUM A. WORK UNIT 137
a. PRIMARY b. CONTRIBUTING c. Contributing CARDS 114(e)(f)				11. TITLE (Pencode with Security Classification Code) (U) Immunological studies of rickettsial proteins		
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^b 003500 Clinical medicine; 004900 Defense; 010100 Microbiology						
13. START DATE 76 02	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house			
17. CONTRACT/GRAANT		18. RESOURCES ESTIMATE				
a. DATES/EFFECTIVE:	EXPIRATION:	FISCAL YEAR	PREVIOUS 76	C. PROFESSIONAL MAN YRS 0.1	b. FUNDS (in thousands) 5.0	
b. NUMBER: ^b NA	c. TYPE:	CURRENT	77	0.5	20.0	
d. KIND OF AWARD:	e. AMOUNT: f. CUM. AMT.					
19. RESPONSIBLE DOD ORGANIZATION		20. PERFORMING ORGANIZATION				
NAME: ^b USA Medical Research Institute of Infectious Diseases ADDRESS: ^b Fort Detrick, MD 21701		NAME: ^b Rickettsiology Division USAMRIID ADDRESS: ^b Fort Detrick, MD 21701				
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Pencil NAME if U.S. Academic institution) NAME: Pedersen, Jr., C. E. TELEPHONE: 301 663-7465 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME:				
21. GENERAL USE Foreign intelligence considered		POC:DA				
22. KEYWORD (Pencode EACH with Security Classification Code) (U) Rickettsiae; (U) Vaccines; (U) Immunogenic proteins; (U) Military medicine; defense BW						
23. TECHNICAL OBJECTIVE, ^b 24. APPROACH, 25. PROGRESS (Pencil individual paragraphs identified by number. Pencode text of each with Security Classification Code.) 23 (U) Isolate and utilize components of tick-borne rickettsiae to manufacture new vaccines designed to protect against disease caused by tick typhus and spotted fever rickettsia of importance for medical defense against BW agents. 24 (U) Isolate purified components of tick-borne rickettsiae; determine antigenic, immunogenic and physicochemical properties. 25 (U) 76 02 - 76 06 - Efforts are currently directed toward the isolation and characterization of the group- and species-specific antigens of members of the tick typhus group. Rate-zonal purified rickettsiae have been analyzed by PAGE and at least 32 proteins have been detected in the prototype Sheila Smith strain of R. rickettsii. After disruption of the microbes by sonic oscillation, a soluble fraction has been separated which contains a major protein component (ca. 60,000 MW). This fraction reacts in gel diffusion and counterimmunoelectrophoretic serologic reactions with hyperimmune rabbit and convalescent rhesus monkey sera. Both the soluble and particulate fractions will be studied for their efficacy in animal protection experiments for potential candidate vaccines. In addition, these components may prove useful in immunologic tests for the rapid diagnosis of tick typhus infections. Development of methodology applicable for propagation, purification, analysis and isolation of reactive subunits is now available in this laboratory for the study of other rickettsiae of military significance. Publication: J. Clin. Microbiol. 2:121-125, 1975.						
*Available to contractors upon contractee's approval.						

BODY OF REPORT

Project No. 3A061101A91C: In-House Laboratory Independent Research
(3A762760A834)

Task No. 3A061101A91C 00:
(3A762760A834 02) Prevention and Treatment of Biological Agent Casualties

Work Unit No. 91C 00 137: Immunological Studies of Rickettsial Proteins
(834 02 306)

Background:

Recently, Obijeski et al.¹ compared selected rickettsiae by SDS-polyacrylamide gel electrophoresis. A minimum of 27 proteins was found and Rickettsia rickettsii and Rickettsia akari were differentiated on the basis of their electropherograms; distinctive differences permitted Rickettsia prowazekii to be distinguished from other rickettsiae. Estimated MW of the proteins ranged between 20,000 and 180,000; however, no glyco- or lipoproteins were detected in gels stained by standard procedures. Tzianabos et al.² reported that of the proteins detected in R. rickettsii, 9 were in extracted group-specific CF antigens.

Progress:

We are currently involved in the concentration, purification and analyses of members of the tick-borne rickettsiae. Procedures utilized involve propagation in primary avian cell cultures (duck or chick) in roller bottles. After 7 days growth in antibiotic-free E-199 medium, cells are subjected to 2 freeze-thaw cycles, removed and homogenized. After slow-speed centrifugation to remove cell debris, rickettsiae are pelleted through a cushion containing 15% sucrose in NaCl-Tris-EDTA (NTE) buffer, pH 8.6. The pellet is suspended; rickettsiae are subjected to rate-zonal centrifugation for 18 hr in 30-60% linear sucrose gradients. The opalescent band at a density of ~1.30 gm/ml is collected, diluted in buffer, and pelleted through a 15% sucrose cushion. Material is then suspended in minimal buffer (~1.5 ml) and stored at -70 C for analysis.

Preliminary results of studies on the composition of the prototype Sheila Smith strain of R. rickettsii indicate that this organism has ~32 proteins which may be detected by discontinuous disc gel polyacrylamide electrophoresis (PAGE) using a 7.5% gel concentration.

Several methods for disruption of the organisms have been examined; the most effective for purposes of isolating immunogenic components of interest is the ultrasound technique. In this simple procedure, sonication of purified rickettsiae is followed by sedimentation of large debris (corpuscular antigen) by rate-zonal centrifugation. While we have concentrated our efforts on the top (soluble) component, we intend to examine the properties of the large MW fraction which pellets through the 15-30% gradient. By electron microscopy,

the top fraction is composed of relatively homogeneous macromolecular components, which correspond to and contain predominantly protein fraction IV (MW 60,000) of the intact organism. We have not as yet determined the exact location of this moiety, although we believe it is associated with the cell wall, due to the ease of separation by ultrasound.

We have utilized gel diffusion and counterimmunoelectrophoresis for the immunologic identification of isolated subunit structures. The top component contains antigens which cross-react in these tests, using hyperimmune rabbit antiserum, with other members of the tick-borne rickettsiae Rickettsia siberica and Rickettsia conorii and the Iowa strain of R. rickettsii. Two sera from humans convalescent from R. rickettsii infection did not react with the antigen; however, the serum of one convalescent rhesus monkey did.

Presentation:

Pedersen, Jr., C. E. Studies on antigenic components of selected alphaviruses: immunogenicity of envelope components. Presented, American Society of Tropic Medicine and Hygiene Meeting, New Orleans, LA, 10-14 Nov 1975.

Publication:

Pedersen, Jr., C. E., L. R. Bagley, R. H. Kenyon, L. S. Sammons, and G. T. Burger. 1975. Demonstration of Rickettsia rickettsii in the rhesus monkey by immune fluorescence microscopy. *J. Clin. Microbiol.* 2:121-125.

LITERATURE CITED

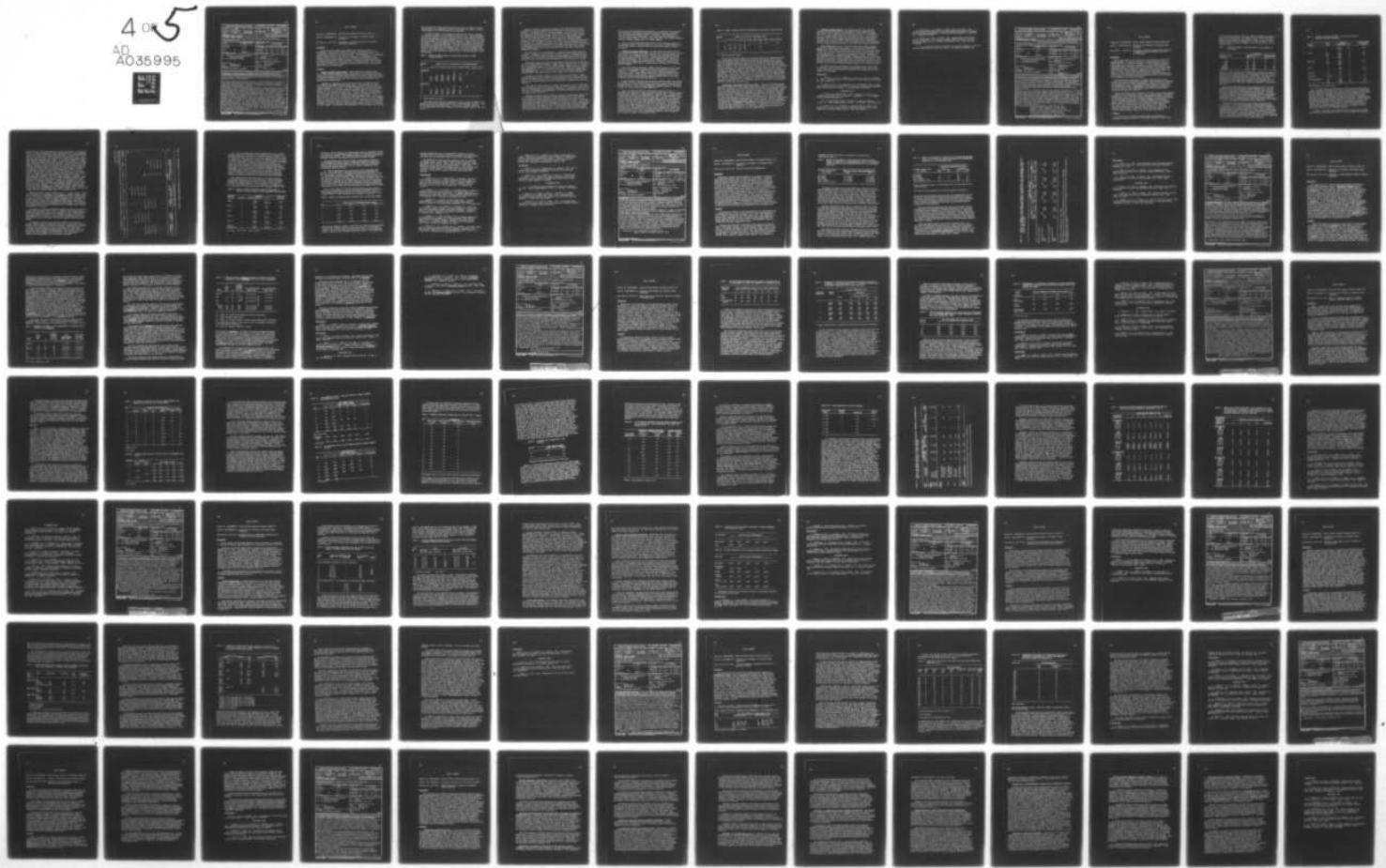
1. Obijeski, J. F., E. L. Palmer, and T. Tzianabos. 1974. Proteins of purified rickettsiae. *Microbios* 11:61-76.
2. Tzianabos, T., E. L. Palmer, J. F. Obijeski, and M. L. Martin. 1974. Origin and structure of the group-specific, complement-fixing antigen of Rickettsia rickettsii. *Appl. Microbiol.* 28:481-488.

AD-A035 995 ARMY MEDICAL RESEARCH INST OF INFECTIOUS DISEASES FR--ETC F/G 6/5
ANNUAL PROGRESS REPORT - FY 1976.(U)
JUL 76 F B ABELES, A O ANDERSON, J B ARENSMAN

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ³ DA 0B6420	2. DATE OF SUMMARY ⁴ 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SURY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCY ⁵ U	6. WORK SECURITY ⁶ U	7. REGRADING ⁷ NA	8. DISSEM INSTRN ⁸ NL	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT
10. NO./CODES: ⁹ a. PRIMARY 62760A	PROGRAM ELEMENT	PROJECT NUMBER 3A762760A834		TASK AREA NUMBER 02	WORK UNIT NUMBER 407		
b. CONTRIBUTING	c. EQUIPMENT CARDS 114(e)(f)						
11. TITLE (Proceed with Security Classification Code) (U) Development of arbovirus vaccines for diseases of military importance							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ¹⁰ 003500 Clinical medicine; 004900 Defense; 010100 Microbiology							
13. START DATE 64 06	14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house			
17. CONTRACT/GRAANT	18. RESOURCES ESTIMATE PRECEDING FISCAL YEAR 76		19. PROFESSIONAL MAN YRS 1.0	20. FUNDS (in thousands) 124			
a. DATES/EFFECTIVE:	EXPIRATION:		21. CURRENT 77	22. PERFORMING ORGANIZATION NAME: Virology Division USAMRIID ADDRESS: Fort Detrick, MD 21701			
b. NUMBER: ¹¹ NA	c. TYPE: d. AMOUNT: e. CUM. AMT.		23. SOCIAL SECURITY ACCOUNT NUMBER:	PRINCIPAL INVESTIGATOR (Name or S/N if U.S. Academic Institution) NAME: Cole, Jr., F. E. TELEPHONE: 301 663-7241			
g. KIND OF AWARD:			24. ASSOCIATE INVESTIGATORS NAME: Rosato, R. R. NAME:				
19. RESPONSIBLE DOD ORGANIZATION NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701	20. RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		25. POC:DA				
21. GENERAL USE Foreign intelligence considered							
22. KEYWORD (Proceed each with Security Classification Code) (U) BW defense; (U) Military medicine; (U) Vaccine development; (U) Arboviruses; (U) Dengue virus; (U) Tick-borne encephalitis							
23. TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Punctuate individual paragraphs identified by number. Proceed each with Security Classification Code.) 23 (U) Develop and produce inactivated and attenuated arbovirus vaccines which may then be combined or administered sequentially to military forces for prophylaxis in geographically or BW oriented ways.							
24 (U) Arboviruses are propagated in primary or certified diploid cell cultures and inactivated with formalin or selected for attenuation. Products are tested for safety and potency in animals. Efficacy is determined by subsequent challenge, or by determination of serological conversion.							
25 (U) 75 07 - 76 06 - Studies have commenced using a newly isolated LGT virus strain to be developed into a live vaccine for prophylactic use against members of the Russian spring-summer encephalitis (RSSE) virus complex. The strain was identified by neutralization tests; high titered working seed virus was prepared. Serial passage in FDA-approved diploid cells (DSB-103) adapted the virus, so that yields of 100 thousand PFU/ml are obtained by day 3 or 4. Prior to initiation of clonal studies, a monkey protection test is now being conducted using the most distantly related virus in the RSSE complex, Powassan, as challenge virus; if protection occurs clonal studies will commence. Initial studies were undertaken on development of a DEN-1 attenuated vaccine. By 6 serial passages in DBS-103 cultures (3 recent human isolates) were adapted to cell culture, yielding 1 million PFU/ml by day 4. Positive identification tests were completed and a variety of necessary high titered seed stocks and antisera prepared. A modified plaque assay and neutralization test system were developed to permit quantitation in 7 days. Neurovirulence tests were completed on an additional lot of WEE vaccine.							
Publications: Milit. Med. 14:163-166, 1976; J. Clin. Microbiol. 3:460-462, 1976.							
Available to contractors upon contractor's approval.							

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 407: Development of Arbovirus Vaccines for Diseases of Military Importance

Background:

Vaccine research programs are typically multifaceted in nature, i.e., development of new products, product improvement, and development of new or alternate procedures are all basic to such a broad research effort. Thus studies were conducted in the following areas: (a) development of an attenuated dengue-1 (DEN-1) vaccine, since none is available; (b) development of an attenuated Langat (LGT) vaccine for use in immunization against the Russian spring-summer encephalitis (RSSE) subgroup of viruses; (c) completion of neurovirulence testing on Lot 1-1974 Western equine encephalomyelitis (WEE) vaccine, Inactivated, Dried; and (d) completion of neurovirulence testing on attenuated DEN-2 vaccine in rhesus monkeys.

Progress:

Studies with DEN-1 Virus Strains. Serum samples were obtained from Dr. L. Rosen, Pacific Research Section, NIAID, NIH, from 3 infected patients. Initial viral isolation attempts using certified green monkey kidney cells from the Lederle Laboratory frozen-cell bank proved to be futile, although the presence of bacterial contamination in one of the 3 original serum specimens was documented during these attempts.

Subsequently, bank-frozen, certified fetal rhesus lung (DBS-103) cells were obtained from Merrell-National Laboratories. Using the DBS-103 cells, virus was isolated from 2 of the 3 human serum samples and then only after prolonged incubation (7 - 20 days). Once the 2 isolates were obtained (designated DEN-1 #2 and DEN-1 #3) they were serially passaged in DBS-103 cultures in an attempt to: (a) increase the titer (e.g., only 10^3 PFU/ml on first outgrowth) and (b) decrease the time to optimal titer. Briefly, replicate 75-cm² cultures were inoculated with 0.4 ml of undiluted, original serum specimen. After adsorption for 1 hr at 35 C, maintenance medium (MM; EMEM + NEAA + 2% certified FBS + P/S) was added and the cultures were incubated at 35 C for 20 days; samples taken at regular intervals were stored at -70 C for subsequent assay once we had developed a reliable, sensitive plaquing method. The 2 strains were serially passaged 6 times using undiluted fluid from each passage harvest as inoculum for the next passage. As required by FDA Regulations¹ similar passages and samplings

were performed with sham-inoculated, replicate cultures of DBS-103 cultures; these samples will ultimately be required to document the absence of adventitious agents once vaccine production is undertaken.

As shown in Table I the 2 strains had adapted sufficiently by passage 5 to produce a maximum of $\sim 10^6$ PFU/ml as early as day 4. Slight differences were noted between the strains in terms of extent of cell destruction (CPE) elicited and the speed with which adaptation to the cells occurred, with DEN-1 #2 being somewhat more pronounced in both instances. Plaques on BS-C-1 cells (see below), both strains exhibited plaques that were similar with regard to size range (1 - 5 mm) and morphology. It should be noted that neither strain elicited even moderate CPE during the course of a normal incubation period and little cell debris was ever apparent, even at the time of maximum virus release. This characteristic, if maintained in the plaque-purified clone(s) selected for vaccine development, should present us with an extremely "clean" final product.

TABLE I. EFFECT OF SERIAL PASSAGE OF REPLICATION OF DEN-1 #2 VIRUS IN DBS-103 CELLS

PASSAGE NO.	PFU/ML ($\times 10^4$) OF ISOLATES BY DAY POSTINOCULATION ^a							
	3	4	5	6	7	10	15	20
<u>Isolate #2</u>								
1					<1		<1	<1
2					26	100	70	
3		27	180	78	60			
4	88	160	230	460	790	640		
5	32	160	610	330	280	280		
6	16	58	100	92	53	88		
<u>Isolate #3</u>								
1					<1		<1	<1
2					<1	20	20	
3		<1	2	5	17			
4	16	78	100	160	310	320		
5	75	180	75	200	200	400		
6	20	100	100	120	260	73		

a. Blank denotes not tested.

Concurrent with isolation and adaptation of the 2 strains to DBS-103 cells an assay procedure was developed using BS-C-1 (monkey kidney) cultures. In our system, plaques can be counted after 7 days and the resultant titers are $\sim 1 - 2 \log_{10}$ higher than those obtained in the more commonly used LLC-MK₂ plaque system.

Having developed a suitable assay system we next concentrated on the development of a plaquing method which would utilize a cell culture that is acceptable by FDA Regulations¹ for vaccine development. Attempts made in WI-38 and primary CEC and DEC cultures were unsuccessful. However, initial attempts with DBS-103 cultures yielded plaques in 10 - 13 days. Unfortunately, after this early success difficulties were encountered in the plaquing procedure. At the time of writing the reason(s) for our inability to produce plaques routinely in these FDA-approved cells is not known. We are currently reevaluating factors such as: (a) degree of "sheet-out" at the time of inoculation; (b) age of cultures at the time of inoculation; (c) effect of different lots of the routine plaquing medium; and (d) the effect of using neutral red stain in the initial overlay medium, rather than at the end of the normal time period required for appearance of plaques.

The seriousness of this deterrent to our dengue vaccine program cannot be overstated. Obviously we will continue to pursue this problem vigorously until resolved. The ability to employ plaque-to-plaque passages in FDA-approved cells is required to: (a) eliminate any possible contaminating agents in the original specimens and (b) ensure the genetic homogeneity of selected clones so treated.

During this year much progress has been made in areas ancillary to the main thrust of dengue vaccine development. Repeat plaque reduction serum neutralization (NT) tests performed both here and at WRAIR (Personal communication, Dr. Brandt) have confirmed the identity of the isolates as DEN-1. These tests were conducted using known DEN-1, -2, -3, and -4 mouse ascitic fluid obtained from WRAIR and employing known DEN-1, -2, -3 and -4 virus strains from the ATCC (working seeds prepared here). Thus identification of our isolates is considered absolute.

The need for reference reagents in this program is self-evident. These include hyperimmune ascitic fluids and sera, and high titered virus preparations made both in mouse brains (for use as HI antigens) and in cell culture (for NT tests). All of these reagents are in the final phase of preparation or have been completed.

High titered stocks of DEN-1, -4 viruses were prepared in BS-C-1 cells and are in routine use in NT tests; similar stocks were also prepared in DBS-103 cells. Further, blind passages in suckling mice (SM) appear to be adapting the virus to that host; we anticipate the availability of SM brain antigens for routine use in serological tests by June 76.

Hyperimmune hamster ascitic fluids are currently being prepared against the two isolates. To obtain sufficient "clean" antigenic mass for this purpose, virus-containing culture fluids were clarified (11,700 g for 30 min) and the virus separated by PEG treatment (10% FCS, 10% PEG-6000, 2.2% NaCl). Virus thus precipitated was pelleted by centrifugation at 11,700 g for 30 min. Pellets were then suspended in HBSS + 2% FCS + antibiotics and purified of additional cellular material by pile centrifugation (10% w/w sucrose over 50% w/w sucrose in TNEBP buffer; #30 rotor, 105,700 g for 2 hr). The band of virions at the interface was removed and diluted sufficiently to permit repeated inoculation of hamsters

with this 30-X concentrated virus preparation. In addition 6 rhesus monkeys were determined to be negative for group B arbovirus antibody, generally (HI tests) and specifically for DEN-1 - 4 (NT test). These animals have been inoculated with a single dose of the 4 DEN serotypes from WRAIR plus 2 of our isolates (DEN-1 #2 and #3) to prepare the most type-specific antibody possible (Personal communication, Dr. Brandt, WRAIR).

All of the above-mentioned reagents will be used routinely during the developmental stages of the DEN vaccine as well as for the FDA-required tests on the final product to document absence of adventitious agents, identity and potency.

Studies with Langat (LGT) Virus. As indicated in last year's report² a decision was reached to discontinue work with clones derived from Dr. Price's LGT vaccine³ and to begin an intense evaluation of a newly isolated strain with a short, well-documented history. Studies were initiated in the fall of 1975 utilizing an isolate (strain T-1674) obtained from Dr. Bancroft of the SEATO Laboratories. The strain had been isolated from a tick and was received as 1st passage mouse brain material. Titration in SM and by the plaque technique showed that the original sample contained $\geq 10^{7.0}$ SMICLD₅₀ and $10^{6.9}$ PFU/ml.

Seed virus stock was prepared in COFAL-tested embryonated chicken eggs (CE slurry) and was shown by plaque titration in MK₂ and CEC cultures to contain $\sim 2 \times 10^8$ PFU/ml. Plaque sizes in this preparation varied from 1-4 mm after 6 days in CEC culture, indicative of the heterogeneity of the strain as received. Virus from the CE slurry was identified as LGT virus by NT tests using antiserum prepared against the M854 strain of LGT virus obtained from the Yale Arbovirus Research Unit.

Serial passage of the virus was conducted in DBS-103 culture (as described for DEN-1) using the SEATO strain as received for inoculation of the first passage cultures. After a single passage the virus adapted to DBS-103 cells in terms of time to peak titer and thereafter regularly produced $\sim 10^5$ PFU/ml by day 3 or 4 with little or no CPE (Table II).

Prior to conducting further in vitro studies with this strain an experiment was initiated to verify the ability of LGT virus to protect monkeys against another member of the RSSE subgroup, Powassan virus. A general lack of confidence in the results published by Dr. Price of Johns Hopkins^{4,5} necessitated this small-scale repeat of his experiments. Briefly, in cooperation with AA Division personnel, 4 rhesus monkeys seronegative to group B arboviruses by HI test and to LGT virus specifically (NT test) were inoculated SC with $\sim 10^6$ PFU of strain T-1674 LGT virus as CE slurry. To provide baseline data the animals were bled on days -1, 0, and days 1-10 and 29 for hematocrit, WBC, RBC, differentials as well as for viremia and serological determinations; temperatures were recorded daily throughout the study. This "immunizing-infection" with LGT virus proved uneventful except for a slight elevation in WBC on day 1 and a corresponding increase in total monocytes; both returned to normal in all animals by day 3.

TABLE II. EFFECT OF SERIAL PASSAGE ON REPLICATION OF LGT VIRUS IN DBS-103 CELLS

PASSAGE NO.	PFU/ML (X 10 ⁴) BY DAY POSTINOCULATION ^a										
	3	4	5	6	7	8	9	10	11	12	15
1					3	2	7	240	18		90
2		10	18	18	16	6			0		
3	<1	1	2	4	14			18			
4	10	10	18	14	12			1			
5	9	15	11	10	3			<1			

a. Blank denotes not tested.

To test the protective efficacy of the LGT infection all 4 immunized monkeys plus 2 seronegative control monkeys were challenged intracerebrally (IC) with $\sim 10^6$ PFU of Powassan virus, the most distantly related member of RSSE subgroup. Using the bleeding schedule described above the animals are currently being evaluated for changes in the above-mentioned parameters and for overt signs of disease. During the course of this study the Biologic Production Suite has been decontaminated to allow performance of necessary maintenance procedures which, at the time of writing, have not been completed. Thus, samples taken from monkeys for viremia and serological determinations must remain locked in freezers until the Suite is again operational. If the study reveals that the new LGT strain protects monkeys against Powassan virus infection as evidenced by: (a) decreased viremia in "immunes" vs. controls after challenge and/or (b) increased immunological response (i.e., "secondary type") to Powassan virus in immunes vs. controls, then studies will be resumed generally as described last year,² including isolation, purification and characterization of clones from the new isolate.

WEE Vaccine, Inactivated, Dried (Lot 1-1974). A group of 20 healthy guinea pigs was obtained for a repeat neurovirulence test of this 2nd lot of vaccine for human use. Subsequent to IC inoculation with final product, reconstituted for human use, the animals were observed for 18 days for signs of neurologic disease; appropriate control animals were also inoculated and observed. The animals were then necropsied and histopathological examination of each brain was made by Pathology Division personnel. No gross lesions were observed in any of the test animals. Only one test guinea pig had CNS lesions characteristic of an infectious agent, multifocal granulomatous meningoencephalitis with multifocal lymphocytic perivascular cuffing of some meningeal, hippocampal and cerebral vessels. However, organisms and pseudocysts of Encephalitozoon cuniculi were present within the lesion and were determined to be the etiology of these lesions and therefore were not vaccine-related (Personal communication, MAJ Whitmire). A submission for this lot of WEE vaccine was recently prepared for the Army Investigation Drug Review Board and forwarded to HQDA for approval to use the product in man.

Studies with DEN-2 Vaccine. In cooperation with AA and Pathology Division personnel the monkey neurovirulence test was conducted on this experimental product prepared by CPT McManus.⁶ Signs of CNS involvement were seen in all 10 monkeys given the experimental product. In comparison: (a) 1 of 5 monkeys given Yellow Fever (YF) Vaccine, U.S.P. showed CNS involvement; (b) all 5 given parent ("nonattenuated") DEN-2 virus exhibited CNS signs; and (c) 2 of 4 monkeys given vaccine from the lot previously administered to volunteers by Dr. Price also showed signs of CNS involvement. The most commonly observed CNS signs in all groups were tremors and paresis.

Histopathological studies on these test and control monkeys somewhat reflected the neurological signs observed. All YF vaccine controls had minimal focal gliosis both in association with needle tracts and other areas of the brain. Focal infiltration of mononuclear cells in the meninges of all controls was also seen. The infiltration was localized mainly around veins with severe, segmental, nonsuppurative inflammation of some. All monkeys receiving DEN-2 vaccine exhibited minimal to moderate gliosis with some areas more severe than others; however, all areas of the brain were affected. Focal meningeal infiltration by mononuclear cells was observed around veins of the meninges and appeared as nonsuppurative vasculitis in some. Generally, lesions were more severe in DEN-2-inoculated monkeys compared to YF vaccine controls.

The adverse results of the monkey neurovirulence test in concert with the apparent success of WRAIR personnel in developing a DEN-2 vaccine from a more recent DEN-2 human isolate has resulted in the termination of further studies on this vaccine.

Publications:

1. Robinson, D. M., A. T. McManus, F. E. Cole, Jr., and C. E. Pedersen, Jr. 1976. Inactivated Mayaro vaccine produced in human diploid cell cultures. Mil. Med. 141:163-166.
2. Cole, F. E. Jr., C. E. Pedersen, Jr., D. M. Robinson, and G. A. Eddy. 1976. Improved method for production of attenuated Venezuelan equine encephalomyelitis (TC-83 strain) vaccine. J. Clin. Microbiol. 3:460-462.

LITERATURE CITED

1. Food and Drug Administration. 1975. Biologics. p. 3-102. Code of Federal Regulations, Title 21, Chapter 1, Subchapter F. U. S. Government Printing Office, Washington, D.C.
2. U. S. Army Medical Research Institute of Infectious Diseases, 1 July 1975. Annual Progress Report, FY 1975. pp. 283-292. Fort Detrick, MD.
3. Price, W. H., I. S. Thind, R. D. Teasdall, and W. O'Leary. 1970. Vaccination of human volunteers against Russian spring-summer (RSS) virus complex with attenuated Langat E5 virus. Bull. WHO 42:89-94.

4. Price, W. H., I. S. Thind, W. O'Leary, and A. H. el Dadah. 1967. A protective mechanism induced by live group B arboviruses against heterologous group B arboviruses independent of serum neutralizing antibodies or interferon. Am. J. Epidemiol. 86:11-27.

5. Price, W. H., and I. S. Thind. 1973. Immunization of mice against Russian spring-summer virus complex and monkeys against Powassan virus with attenuated Langat E5 virus. Duration of protection. Am. J. Trop. Med. Hyg. 22:100-108.

6. U. S. Army Medical Research Institute of Infectious Diseases, 1 July 1974. Annual Progress Report, FY 1974, pp. 211-214. Fort Detrick, Frederick, MD.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ⁹ DA 0B6423	2. DATE OF SUMMARY ⁹ 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMRY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SECY ⁹ U	6. WORK SECURITY ⁹ U	7. REGRADING ⁹ NA	8. CHG'DN INSTN'R NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES: ⁹	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	62760A	3A762760A834		02	411		
b. CONTRIBUTING							
c. FOREIGN	CARDS 114(e)(f)						
11. TITLE (Pencile with Security Classification Code) ⁹ (U) Evaluation of promising compounds for antiviral use against diseases of medical importance to the military							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁹ 003500 Clinical medicine; 004900 Defense; 010100 Microbiology							
13. START DATE 70 12	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house				
17. CONTRACT/GRANT	18. RESOURCES ESTIMATE	19. PROFESSIONAL MAN YRS	20. PERFORMING ORGANIZATION			21. FUNDS (in thousands)	
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c. TYPE:	4. AMOUNT:			ADDRESS: Fort Detrick, MD 21701			
d. KIND OF AWARD:	5. CUM. AMT.			PRINCIPAL INVESTIGATOR (Punish DSN II U.S. Academic Institution)			
22. RESPONSIBLE DOD ORGANIZATION				NAME: Stephen, E. L.			
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RESPONSIBLE INDIVIDUAL				ASSOCIATE INVESTIGATORS			
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23. GENERAL USE Foreign intelligence considered							
24. KEYWORDS (Pencile EACH with Security Classification Code) ⁹ (U) BW defense; (U) Military medicine; (U) Chemotherapy; (U) Togaviruses; (U) Arenaviruses; (U) Metabolism; (U) Bioavailability; (U) Monkeys							
25. TECHNICAL OBJECTIVE, ⁹ 26. APPROACH, 27. PROGRESS (Punish individual paragraphs identified by number. Pencile text of each with Security Classification Code.)							
23 (U) Evaluate in subhuman primates potential antiviral compounds with significant antiviral activity against infectious diseases in lower animals. The experimental disease models selected for study are important human pathogens of military concern, and the information obtained will be invaluable in control and treatment of viral diseases in military personnel, emphasizing those of possible BW importance.							
24 (U) Test candidate compounds in tissue culture and laboratory animals against selected viruses.							
25 (U) 75 07 - 76 06 - New mouse models have been defined for evaluation of antiviral compounds using yellow fever (YF), Japanese encephalitis and Tacaribe viruses. Dose-response experiments have shown 0.3 mg/kg lysine-stabilized poly I:poly C to be nearly as effective for induction of interferon in rhesus monkeys as 3.0 mg/kg and that 1.0 mg/kg is as effective in treating YF infection of monkeys as 3.0 mg/kg. These data will be utilized to develop and test the most efficacious regimen of therapy. Ribavirin was effective in the treatment of influenza infection of squirrel monkeys when initiated either early after exposure to virus or late after the onset of clinical symptoms. In vitro studies have shown ribavirin to be an effective antiviral agent against YF and Machupo viruses.							
Publications: Programs and Abstracts, ICAAC, No. 247, 1975. Antimicrob. Agents Chemother. 8:154-158, 1975. Abstracts, Am. Soc. Microbiol., p. 5, 239, 1976. Program, 3rd Conf. Antiviral Substances, No. 24, 1976. Toxicol. Appl. Pharmacol. 35:107-111, 1976. J. Infect. Dis. 133 (suppl.):A140-A144, 1976. Army Science Conference Proc. I:263-271, 1976							

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 411: Evaluation of Promising Compounds for Antiviral Use Against Diseases of Medical Importance to the Military

Background:

Vaccine prophylaxis is not effective for the prevention of all virus-induced disease. In addition, vaccines are usually virus-specific and ineffective after the onset of infection. For these reasons, chemotherapeutic agents for prevention or treatment of virus-induced disease are critically needed. Previous reports¹ have described the successful chemo-prophylaxis and early treatment of model infections of mice caused by VEE and YF viruses. Studies have been completed to characterize further the theory of indirect mouse models and describe their usefulness. In addition, mouse models have now been developed to evaluate candidate anti-viral agents for use against Japanese encephalitis (JE) virus and Tacaribe virus (a member of the arenavirus group).

The dose-response relationship for lysine-stabilized poly I·poly C (PICLC)² has not been investigated in monkeys. This information is necessary for the development of cogent recommendations relative to the most efficacious regimen of therapy. Experiments have been completed to assist in the formulation of these recommendations. The limits of the prophylactic efficacy of PICLC and its effectiveness against VEE and Machupo virus infections have also been evaluated.

Vaccine prophylaxis against influenza virus (New Jersey strain) infection is expected to be inadequate during the next influenza season. Insufficient supply of the current antigenic strain combined with its uncertain efficacy are contributing factors. The successful treatment of influenza infections of the mice have been extended to a newly developed squirrel monkey model. Successful chemotherapy of influenza infection in this subhuman primate model is expected to be quite relevant to similar infections in man. The in vitro sensitivity of several viruses to the antiviral activity of human interferon has been characterized in a variety of tissue culture systems. In addition, the in vitro antiviral activity of ribavirin against Machupo, VEE, and YF viruses has been defined.

Progress:

I. Validity of the indirect YF mouse model¹ was evaluated by attempting to measure alteration in the viremia response of treated mice

vs. untreated mice given YF virus SC. Since no viremia was detected in inoculated, untreated mice, this approach did not resolve the interpretive problem inherent in the indirect concept. As an alternative approach, the immunizing challenge dose of virus was varied, using a constant dosage of PICLC (3.0 mg/kg). The results of this study are shown as Table I.

TABLE I. FURTHER ASSESSMENT OF THE SPECIFICITY OF THE INDIRECT YF MOUSE MODEL.

IMMUNIZING DOSE, PFU/mouse	SURVIVAL			
	Untreated		PICLC-Treated	
	S/25	%	S/25	%
10	4	16	0	0
100	15	60	0	0
1,000	23	92	2	8
10,000	25	100	3	12
100,000	24	96	9	36
1,000,000	25	100	19	74

Clearly, 10 PFU of virus were not sufficient to establish an immunizing infection in the untreated mice. PICLC inhibited virus replication except when the immunizing dose was > 100,000 PFU. These data confirm previous observations that challenge dose of virus is a critical determinant in the description of a successful model infection. The dose-response relationship lends credence to the assumption that an indirect approach to evaluation of antiviral compounds is both specific and valid.

We have previously shown that 42-day-old mice can be killed with Tacaribe virus if they are injected intracerebrally (IC). Tacaribe virus is not lethal by peripheral routes. Further, mice given the virus SC 21 days prior to IC challenge can be protected. Therefore, an indirect Tacaribe mouse model was developed to assess the potential of various compounds to alter arenavirus infections. The compounds evaluated in the 1st study were not sufficiently active to prevent replication of the immunizing dose of virus, since the mice were immune to a 2nd IC challenge of the homologous virus (Table II).

Ribavirin was only effective at toxic doses (100 mg/kg killed 21 of 30 mice). The recent development of a mouse model permitted a preliminary study to evaluate the antiviral potential of compounds previously shown to be active against YF virus. PICLC, kethoxal, and rimantadine were evaluated in this study. PICLC was the only compound that increased survival. These data combined with the data from the Tacaribe mouse model, support the supposition that a specific antiviral effect is manifested by the indirect YF mouse model since the compounds were not uniformly effective in these model infections.

TABLE II. INDIRECT TACARIBE MOUSE MODEL TO ASSESS THE ANTVIRAL ACTIVITY OF SEVERAL COMPOUNDS.

COMPOUND	DOSE, mg/kg	MORTALITY		TIME TO DEATH Days
		Dead/Total	%	
0		20/30 0/30 ^a	67 0	8.6 -
11,567	250	2/26	8	9.0
	125	1/30	3	9.0
	63	0/29	0	-
	31	0/29	0	-
Mepacrine	125	2/23	9	14.5
	63	0/26	0	-
	31	0/28	0	-
PICLC	3	1/27	4	6.0
	1	1/30	3	7.0
Kethoxal	60	0/29	0	-
Ribavirin	100 mg	3/9	33	7.0
Bis-benzimidazole	25	1/26	4	7.0
	60	3/20	15	12.0
	20	0/29	0	-

^a10³ PFU/mouse, SC, Tacaribe virus.

II. The dose response of monkeys given various doses of PICLC revealed a margin of safety of at least 20. Since the minimum dose (0.3 mg/kg) stimulated a significant interferon (IF) response and the maximum dose (6.0 mg/kg) did not produce any untoward side effects, the absolute margin of safety cannot be resolved. The maximum IF response in all groups of monkeys, regardless of dose, occurred after the 2nd PICLC injection. Hyporesponsiveness was noted following the 3rd injection and continued to the 6th injection. The monkeys regained approximately 50-70% of the maximal IF response from the 6th - 10th injection. It is hoped that hyporesponsiveness may be circumvented by initiating therapy using a dose of 0.3 mg/kg followed by larger doses during the hyporesponsive period, and returning to the low dose in the later part of the treatment regimen.

In order to investigate the feasibility of reducing the dose of PICLC and to evaluate lower challenge doses of YF virus, monkeys were challenged

with either 10 or 1,000 PFU/monkey and treated with either 1 or 3 mg/kg of PICLC. In previous experiments using a challenge dose of 1,000 PFU/monkey, the time to onset of viremia was invariably on day 2 and the time to death was 4.8 ± 0.4 (SE) days. Monkeys given 10 PFU of YF (Table III) had a delay in onset of viremia and a longer time to death than untreated monkeys given the same challenge dose of virus. It is unclear whether the longer time course in monkeys given 10 PFU is related to the decrease in challenge dose, since the untreated control monkey given 1,000 PFU also had a longer course of illness. In previous work with YF in rhesus monkeys (Kosch and Spertzel, unpublished data), the initial challenge dose of virus appeared to affect the time to onset of illness, but not the duration. Monkeys treated with 1.0 mg/kg of PICLC and challenged with 10 PFU of YF had a 5-6 day delay in onset of viremia, and 1 of 3 monkeys was not detectably viremic. In addition, there was a marked reduction in peak virus concentration. The delay in onset of viremia was not so apparent in monkeys challenged with 1,000 PFU of YF. The viremia responses of monkeys treated with 3.0 mg/kg of PICLC were similar to those of monkeys given 1.0 mg/kg. Therefore, 1.0 mg/kg of PICLC is as effective as 3.0 mg/kg in the prophylaxis of YF disease in monkeys. In addition, decreasing the challenge dose of virus, at least in PICLC-treated monkeys, appears to yield a more protracted disease syndrome. It is possible that later treatment would be of value if the challenge dose of virus were reduced, since 1,000 PFU of YF may represent $\geq 10,000$ monkey lethal doses of virus. All of the surviving monkeys had neutralizing antibody titers of $\geq 1:320$ by day 42.

Monkeys were given 1 dose of PICLC at -6, -2, -1, 0 or +1 days to investigate the prophylactic potential of this compound. Monkeys treated on day -6 or -2 died with no differences from untreated controls. Monkeys treated on day -1 or 0 had a 2-day delay in onset of viremia, and 1 of 3 monkeys in each group survived. Two of 3 monkeys treated on day +1 survived. The delay in onset of viremia and the survivors in the latter groups suggest that PICLC has potential as a prophylactic antiviral agent. It is possible that multiple doses at various intervals prior to challenge might increase the prophylactic potential.

The response of VEE-infected monkeys treated with PICLC was reevaluated using a lower challenge dose of virus. In addition, the monkeys were restrained in cages and no catheters were implanted. None of these monkeys in this study died and only 50% (2 of 4) developed detectable SN antibody. The possibility of an antiviral effect of PICLC is suggested by the latter observation, since all untreated, VEE-infected monkeys developed SN antibodies.

A preliminary experiment was conducted in Machupo virus-infected monkeys. Machupo virus is relatively insensitive to the effects of interferon in vitro, compared with VEE and YF viruses. Treatment was initiated at various times including 8 hr prior to infection and 7 days after infection. Treated monkeys had significantly greater viremia titers and died earlier than untreated, infected monkeys. The results of this preliminary experiment suggest that PICLC should not be used against viruses that are insensitive to the effects of interferon.

TABLE III. DOSE RESPONSE OF PICLC IN YT-INFECTED RHECUS MONKEYS (3/GROUP)

DAY	LOG 10 PFU/ml OF VIRUS \pm SEM					
	Untreated Control 10 PFU (n=1)	1.0 mg/kg PICLC		3.0 mg/kg PICLC		
		10 PFU	1,000 PFU	10 PFU	1,000 PFU	
0-2	-	-	-	-	-	-
3	2.5 \pm 0.43	4.3	-	-	-	-
4	6.7 \pm 0.48	6.7	-	-	-	-
5	8.7 + 0.33	8.3	-	2.7 \pm 1.00	-	2.7 \pm 0.67
6	7.9 \pm 0.24	8.0	-	4.1 \pm 1.20	-	3.4 \pm 1.27
7	Dead	8.2	-	4.7 \pm 1.93	2.1 \pm 0.43	2.4 \pm 0.66
8	Dead	2.7 \pm 1.00	2.1 \pm 0.39 ^b	3.1 \pm 1.43	2.8 \pm 1.15 ^b	
9	3.3 \pm 1.05	-	4.0 \pm 1.36	-	-	-
10	3.2 \pm 1.31	-	4.9 \pm 1.12	-	-	-
11	3.3 \pm 1.57	-	3.1 \pm 1.42 ^b	-	-	-
12	1.8 \pm 0.10	-	-	-	-	-
13-17	-	-	-	-	-	-
Survival	0/3	0/1	3/3	2/3	2/3	2/3

^aMean titers were calculated using a value of 1/2 the lowest detectable viremia, or 50 PFU/ml for negative titers.

III. Previous studies have shown rimantadine HCl to be a potent antiviral drug in the treatment of mice infected with influenza A by a mechanism potentially unrelated to a specific antiviral property, since no reduction of peak lung virus titers or histopathology has been shown in treated mice. Rimantadine is known to be antiviral against influenza A, but not influenza B, in tissue culture systems; therefore, the demonstration of protection of influenza B-infected mice would be an indication that any emphasis toward determining specific mechanism of action should be directed toward potentially stimulated host defense mechanisms. Since no protection of influenza B-infected mice resulted from rimantadine treatment by either the IP or aerosol route, the protective effects noted in previous studies must be of a specific nature. The effect of rimantadine therapy on the functional lung capacity of influenza-infected mice is presently being investigated.

Even though ribavirin has been shown to be a potent antiviral drug for use against influenza virus infections of mice and monkeys, a preliminary experiment was conducted to assess certain structural analogs for increased efficacy. The compounds were all given to mice in a continuous aerosol for 22 hr, beginning 6 hr postinfection. It is clear that 3 of the analogs (Table IV), designated 3086-5, 5970,

TABLE IV. EFFECT OF VARIOUS STRUCTURAL ANALOGS OF RIBAVIRIN ON SURVIVAL AND TIME TO DEATH OF MICE CHALLENGED WITH INFLUENZA.

COMPOUND (ICN NO.)	TREATED	SURVIVAL		MEAN TIME TO DEATH
		Survivors/Total	%	
3086-5	-	5/40	12.5	7.9
	+	32/40	80.0***	8.9
5005-4	-	8/40	20.0	7.6
	+	3/40	7.5	6.9*
5970	-	2/40	5.0	7.7
	+	19/40	47.5***	9.0
5158-4	-	3/39	7.7	7.2
	+	5/39	12.8	8.0
3142-4	-	1/40	2.5	7.3
	+	23/40	57.5***	9.0**
1229-28 (Ribavirin)	-	2/38	5.3	7.8
	+	17/39	43.6***	8.1

*P < 0.05. **P < 0.01. ***P < 0.005.

and 3142-4, were as effective as ribavirin since they all increased survival. In addition, the tri-o-acetyl derivative (3086-5) of ribavirin is more active than the parent compound. This compound requires *in vivo* activation and may represent a means of circumventing the need for continuous therapy.

The demonstration that ribavirin given in small-particle aerosols to influenza-infected squirrel monkeys successfully thwarted the infection when initiated early after exposure to the virus (6 hr) and reversed the syndrome when initiated after the onset of clinical illness was a most exciting and important finding.

IV. Other investigators have reported that YF is inactivated when stored at low pH for 24 hr,³ that many serum components precipitate at pH 2.0, and that IF may not be completely stable at this low pH for 7 days. For these reasons a study was completed to evaluate the time course of inactivation of VEE and YF viruses. Duplicate samples containing 2000 PFU/ml were acidified to pH 2.0 or 3.0 and held at 4°C for up to 7 days. Total inactivation of both VEE and YF viruses occurred 15 min after acidification at either pH. Untreated control virus serum samples showed no significant loss of infectivity. Recent studies have shown a more protracted time for inactivation at pH 3.0 when the virus inoculum was increased to $10^{7.0}$ PFU/ml.

The *in vitro* activity of ribavirin against Machupo virus was investigated in collaboration with the Virology Division (Work Unit 834 03 405). This compound was extremely effective in reducing the yield of virus at 32 µg/ml (Table V). This concentration of ribavirin is achievable *in vivo*

TABLE V. EFFECT OF RIBAVIRIN LEVEL ON MACHUPO VIRUS IN VERO CELL CULTURE

DAY	LOG ₁₀ PFU/ml BY RIBAVIRIN DOSAGE (µg/ml)			
	0	3.2	10	32
3	5.88	5.43	4.27	1.17
4	6.17	5.59	4.48	0.87
5	6.00	5.55	5.08	1.30
6	5.81	5.57	5.52	1.92
7	5.10	4.95	5.09	<2.0

using a daily dosage of 20 mg/kg. Ribavirin was further evaluated in tissue culture using a standard yield reduction assay with VEE, YF and Machupo viruses. Ten, 25 and 60 µg/ml of ribavirin all effectively decreased the growth of Machupo virus confirming the previous results. The compound had

no effect against VEE virus, but inhibited the growth of YF virus. The yield of YF was decreased significantly even when the drug contact period was from 3-6 days (virus titer on day 3 was ~ 10^7 PFU/ml).

The sensitivity of VEE, vesicular stomatitis (VS), YF, Tacaribe, and Parana viruses to the effects of human IF were evaluated in Vero and LLC-MK₂ tissue culture. Parana, VEE, and VSV viruses were equally sensitive to IF in the Vero cell culture system. On MK₂ cells, VEE and VSV viruses were equally sensitive and 2 x more sensitive than YF and/or Tacaribe viruses. YF and Tacaribe viruses were equally sensitive. Parana did not plaque on MK₂ cells and could not be evaluated.

Presentations:

1. Walker, J. S., E. L. Stephen, and R. O. Spertzel. The use of small-particle aerosols of antiviral compounds for the treatment of type A influenza pneumonia in animal models. Presented, Symposium on Antivirals with Clinical Potential, Stanford University School of Medicine, Stanford, Calif., 26-29 August 1975. (J. Infect. Dis. In press.)
2. Stephen, E. L., J. S. Walker, J. W. Dominik, and R. O. Spertzel. Therapeutic effects of ribavirin given by the intraperitoneal and aerosol routes in influenza-infected mice. Presented, 15th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, D.C., 24-26 September 1975. (Programs and Abstracts, no. 247).
3. Stephen, E. L. Advances in viral chemotherapy. Presented, Annual Meeting of the Veterinary Medical Section, Association of Military Surgeons of the United States, Washington, D.C., 13 December 1975.
4. Stephen, E. L., J. S. Walker, J. W. Dominik, H. W. Young, and R. F. Berendt. Aerosol therapy of influenza infections of mice and primates with rimantidine, ribavirin and related compounds. Presented, 3rd Conf. Antiviral Substances, NY Academy of Sciences, New York, N. Y., 2-5 February 1976. Program, no. 24.
5. Stephen, E. L. First successful use of a chemical compound for the prophylaxis and treatment of a lethal, systemic, viral infection common to man and subhuman primates. Presented, 1976 Army Science Conference, U. S. Military Academy, West Point, NY, 22-25 June 1976. (Army Science Conference Proceedings, in press.)
6. Kuehne, R. W., W. L. Pannier, and E. L. Stephen. An indirect mouse model for evaluating potential antiviral compounds. Presented, 76th Annual Meeting, American Society for Microbiology, Atlantic City, N. J., 2-7 May 1976. (Abstracts of the Meeting-1976, p.5.)

7. Sammons, M L., W. L. Pannier, H. B. Levy, S. Baron, and E. L. Stephen. Serum interferon response in rhesus monkeys to modified polyriboinosinic-polyribocytidyllic acid complex. Presented, 76th Annual Meeting, American Society for Microbiology, Atlantic City, N. J., 2-7 May 1976. (Abstracts of the Meeting - 1976, p. 239).

Publications:

1. Rosato, R. R., E. L. Stephen, and W. L. Pannier. 1976. Dose-response data for toxiferine dichloride in monkeys and guinea pigs. *Toxicol. Appl. Pharmacol.* 35:107-111.
2. Stephen, E. L., J. W. Dominik, J. B. Moe, R. O. Spertzel, and J. S. Walker. 1975. Treatment of influenza infection of mice using rimantadine hydrochlorides by the aerosol and intraperitoneal routes. *Antimicrob. Agents Chemother.* 8:154-158.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. July 1974. Annual Progress Report FY 1975. pp. 294-306. Fort Detrick, Md.
2. Levy, H. B., G. Baer, S. Baron, C. E. Buckler, C. F. Gibbs, M. J. Iadarola, W. T. London, and J. Rice. 1975. A modified polyriboinosinic-polyribocytidyllic acid complex that induces interferon in primates. *J. Infect. Dis.* 132:434-439.
3. Wheelock, E. F., And W. A. Sibley. 1965. Circulating virus, interferon and antibody after vaccination with the 17-D strain of yellow-fever virus. *N. Engl. J. Med.* 273:194-198.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ¹ DA OD6415	2. DATE OF SUMMARY ² 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SURVY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY ³ U	6. WORK SECURITY ⁴ U	7. REGRADING ⁵ NA	8. DA DISCH INSTN ⁶ NL	9. CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES ⁷ a. PRIMARY 62760A	PROGRAM ELEMENT	PROJECT NUMBER 3A762760A834		TASK AREA NUMBER 02	11. SPECIFIC DATA 12. LEVEL OF SURV A. WORK UNIT 415	
b. CONTRIBUTING	c. CONFIDENTIALITY CARDS 114(e)(f)					
13. TITLE (Punctuate with Security Classification Code) (U) Studies in respiratory immunization						
14. SCIENTIFIC AND TECHNOLOGICAL AREA ⁸ 003500 Clinical medicine; 004900 Defense; 010100 Microbiology						
15. START DATE 72 07	16. ESTIMATED COMPLETION DATE CONT	17. FUNDING AGENCY DA	18. PERFORMANCE METHOD C. In-house			
19. CONTRACT/GRAANT		20. RESOURCES ESTIMATE	21. PROFESSIONAL MAN YRS	22. FUNDS (\$ in thousands)		
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b. NUMBER: NA		YEAR	76	1.0	158.0	
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e. KIND OF AWARD:	f. CUM. AMT.					
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NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		NAME: Aerobiology Division USAMRIID ADDRESS: Fort Detrick, MD 21701				
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Punctuate with U.S. Academic Institution) NAME: Scott, G. H. TELEPHONE: 301 663-7453 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME:				
25. GENERAL USE Foreign intelligence considered		POC:DA				
26. APPROACHES (Punctuate with Security Classification Code) (U) Therapy; (U) BW defense; (U) Military medicine; (U) Respiratory disease; (U) Aerosols; (U) Particle size; (U) Immunoprophylaxis; (U) Animal models						
27. TECHNICAL OBJECTIVE, 28. APPROACH, 29. PROGRESS (Punctuate individual paragraphs identified by number. Punctuate rest of each with Security Classification Code.)						
23 (U) Investigate basic mechanisms of aerogenic immunization against respiratory infections. Most BW agents are transmissible in aerosols. Since this is the most feasible agent dissemination method for a large-scale covert BW operation, the need for establishing immune defenses in the respiratory tract make this work unit essential in a comprehensive BW defense program.						
24 (U) Respiratory pathogen vaccines attenuated by adaptation to growth at suboptimal temperatures are used as model systems to study the genesis of immunity for protection against respiratory infections.						
25 (U) 75 07 - 76 06 - Studies confirmed that prior immunological experiences with either heterologous or homologous influenza viruses, which resulted primarily in production of humoral antibody, did not adversely affect the immunogenicity of an attenuated, temperature-sensitive influenza vaccine virus subsequently administered to mice by the aerosol route. The temperature-sensitive virus failed to replicate in lungs of mice with homologous humoral antibody. However, in spite of the humoral antibody, virus replication in the upper respiratory tissues provided sufficient antigen to evoke synthesis of secretory antibody in the respiratory tract and ensured protection (98% survival) against respiratory challenge with virulent influenza that killed 93% of the nonimmunized animals.						
Publications: Ph.D. Dissertation, U. Maryland, 1975. Infect. Immunity 13:696-703, 1525-1527, 1976.						

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1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

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BODY OF REPORT

Project No. 3A762760A834: Medical Defense Aspects of Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 415: Studies in Respiratory Immunization

Background:

Significant advances toward elaborating the mechanisms of immunity to viral respiratory diseases have been made in recent years. Antibodies synthesized and secreted locally in the respiratory tract, as well as humoral antibodies and factors not directly associated with antibodies, have been shown to contribute to resistance against infections caused by influenza and other respiratory disease viruses. There is convincing evidence that resistance to influenza infection is better correlated with the level of antibody present in the respiratory tract than with the amount of circulating antibody. Production of this local antibody can be stimulated best by depositing antigen directly in the respiratory tract by means of aerosols, but subsequent viral replication appears necessary to achieve effective antigen concentrations.¹ Thus, live, attenuated virus vaccines have obvious potential in respiratory immunization procedures² and several candidate influenza vaccines have been developed. However, since the general population has experienced previous infections with serologically related types, it is important to ascertain the influence of any partial immunity engendered by previous contacts on the immune response of individuals to live vaccine virus administered by the aerosol route.

Progress:

We examined the effects of prior immunization with 2 heterologous and 1 homologous strains of type A influenza virus on the immunogenicity of a live, attenuated recombinant influenza virus in mice. The recombinant vaccine virus, designated ts-1[E], was derived from a mixed infection in bovine kidney cells with wild type influenza A/Hong Kong/68 (H3N2) and A/Great Lakes/65 (H2N2) ts-1 mutant virus.³ The recombinant virus has a restrictive replication temperature of 38 C.

Groups of mice were first inoculated IP with either the A0/PR/8/34 (HON1), A1/Mal/302/54 (H1N1), or the A2/Aichi/2/68 (H3N2) strain of live virus, or a placebo consisting of sterile allantoic fluid. After 28 days 10^4 EID₅₀ of the ts-1[E] (H3N2) vaccine virus were administered to each group in small (2 μ m) aerosol particles. The lungs and upper respiratory tissues (URT) of individual mice from each test group were assayed for ts-1[E] virus at selected intervals after exposure. The incidence of

infection and peak virus titers for samples from each group are summarized in Table I.

TABLE I. EFFECTS OF PRELIMINARY IP INOCULATION WITH VARIOUS INFLUENZA STRAINS ON INFECTION RATES AND RESPIRATORY VIRUS LEVELS FOLLOWING AEROSOL VACCINATION WITH TEMPERATURE-SENSITIVE (ts), ATTENUATED, LIVE VIRUS

INFLUENZA STRAIN GIVEN BY IP ROUTE	MEAN LOG ₁₀ EID ₅₀ TITER (INFECTED/TOTAL)	
	Lung	URT
PR8 (HON1)	5.0 (9/10)	3.0 (6/10)
Mal (H1N1)	4.1 (9/10)	4.0 (8/10)
Aichi (H3N2)	3.0 (1/10)	5.0 (5/10)
None, allantoic fluid	>4.0 (9/10)	>4.0 (10/10)

Parenteral inoculation with the A0 (HON1) and A1 (H1N1) strains not bearing hemagglutinin (H) or neuraminidase (N) antigens common to those of the ts-1[E] mutant (H3N2) had minimum effect on the incidence of lung infection or replication of ts virus in the lungs. When given the more closely related A2 (H3N2) strain parenterally 28 days before exposure to aerosols of the ts vaccine virus, the lungs of only 1 of 10 mice became infected and the lung-virus titer was lower than observed in mice that received the placebo IP followed by exposure to ts virus. However, the URT of 50% of these mice were infected with the ts vaccine virus; virus replication in these tissues was the same as that observed in normal mice.

Ten mice were killed 28 days after IP inoculation alone and 10 after IP inoculation followed by aerosol exposure to the ts mutant. Blood was collected and bronchoalveolar washings (BAW) were taken and concentrated 10-fold. All samples were pooled and titrated for neutralizing antibody against mouse virulent A2 (H3N2) strain influenza virus (Table II). Neutralizing activity was present in the sera and BAW of mice from all groups that were exposed to aerosols of the ts vaccine virus regardless of their previous exposure history. Sera and BAW antibody levels in mice previously inoculated IP with the homologous A2 strain were no lower than in mice that received only the ts vaccine virus by the respiratory route. Although the data are not conclusive, neutralizing BAW titers in mice previously inoculated with the heterologous A0 (HON1) and A1 (H1N1) strains were lower than in mice given only the aerosol vaccination with ts virus.

Mice immunized by each method were challenged 28 days postvaccination with 10^{2.0} respiratory LD₅₀ of mouse-virulent A2 strain virus, a dose that kills untreated control mice within 8 days. Rechallenge infected the lungs and URT of all parenterally-immunized mice and virus replication was similar to that in nonimmunized mice (Table III). However, 90% of those

TABLE II. EFFECTS OF PRELIMINARY IP INOCULATION ON NEUTRALIZING ANTIBODY TITERS OF MICE FOLLOWING AEROSOL VACCINATION WITH TEMPERATURE-SENSITIVE (ts), LIVE INFLUENZA VIRUS

INFLUENZA STRAIN GIVEN BY IP ROUTE	RECIPROCAL NEUTRALIZING TITER ^a			
	None		ts mutant ^b	
	Sera	BAW	Sera	BAW
PR8 (HON1)	0	0	80	<5
Mal (H1N1)	0	0	60	7
Aichi (H3N2)	>80	0	>80	20
None, allantoic fluid	0	0	>80	14

^aMeans of duplicate assays of pooled samples from 10 mice taken 28 days postvaccination.

^b 10^4 EID₅₀ of virus administered 28 days following IP inoculation.

inoculated IP with homologous virus survived the challenge while no protection was derived from IP inoculation with the heterologous strains. In contrast, both the rate of infection and replication of the challenge virus in tissues from groups vaccinated with ts virus, either with or without previous IP immunization, were lower than in normal mice and essentially all mice survived.

Obviously, previous immunologic experience with influenza viruses, including those with homologous antigenic constituents, did not prevent a subsequent immune response to the ts-1[E] vaccine virus. Replication of the vaccine virus in URT tissues was sufficient to enhance respiratory antibody levels and protection against reinfection. The impaired ability of the attenuated vaccine virus to infect and replicate in the lungs, but not the URT of mice parenterally immunized with homologous virus, suggests that humoral antibody exerts greater influence on lung infections than on infections of the URT.

These experiments complete our work with influenza virus. In accordance with administrative guidance the research plan for this work unit is being rewritten to reflect studies on respiratory melioidosis. Appropriate veterinary and Public Health Service permits required for conducting research with Pseudomonas pseudomallei have been obtained. Two lyophilized strains have been obtained from ATCC and are currently being passed in mice and hamsters to enhance their virulence.

TABLE III. EFFECTS OF COMBINED IP AND AEROSOL VACCINATION ON INFECTION RATES, RESPIRATORY TISSUE VIRUS LEVELS, AND SURVIVAL OF MICE FOLLOWING AEROSOL CHALLENGE WITH VIRULENT INFLUENZA VIRUS (AICHI STRAIN, H3N2)

	VIRUS TYPE GIVEN IP						None	
	A0		A1		A2			
	Virus titer ^a	Incidence	Virus titer	Incidence	Virus titer	Incidence		
With ts vaccination^b								
% survival; n=40	100		100		98		98	
Lung	5.6	6/10	5.7	10/10	4.1	6/10	5.6	
URT	4.3	3/10	4.7	3/10	4.0	6/10	4.0	
Without ts vaccination^c								
% survival; n=40	12		10		90		7	
Lung	8.2	10/10	7.2	10/10	7.1	10/10	8.2	
URT	5.7	10/10	6.5	10/10	5.4	10/10	5.6	

^aMean titers of infected mice; log₁₀ EID₅₀.

^bMice received no ts virus but received the indicated virus IP 28 days prior to challenge.

^cMice received ts virus as aerosol 28 days prior to challenge and 28 days after receiving the indicated virus strains by the IP route.

Publications:

1. Scott, G. H. 1975. Immune responses of mice following sublethal influenza virus infections. Ph.D. Dissertation, University of Maryland, Baltimore, MD. 72 p.
2. Scott, G. H., and R. J. Sydiskis. 1976. Responses of mice immunized with influenza virus by aerosol and parenteral routes. Infect. Immun. 13:696-703.
3. Scott, G. H., and J. S. Walker. 1976. Immunoglobulin-bearing cells in lungs of mice infected with influenza virus. Infect. Immun. 13:1525-1527.

LITERATURE CITED

1. Scott, G. H., and R. J. Sydiskis. 1976. Responses of mice immunized with influenza virus by aerosol and parenteral routes. Infect. Immun. 13:696-703.
2. Jemski, J. V., and J. S. Walker. 1976. Aerosol vaccination of mice with a live, temperature-sensitive recombinant influenza virus. Infect. Immun. 13:818-824.
3. Murphy, B. R., E. G. Chalhub, S. R. Nusinoff, and R. M. Chanock. 1972. Temperature-sensitive mutants of influenza virus. II. Attenuation of ts recombinants for man. J. Infect. Dis. 126:170-178.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^b DA 0D6419	2. DATE OF SUMMARY ^b 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SURVEY 75 11 12	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY ^b U	6. WORK SECURITY ^b U	7. REGRADING ^b NA	8. DISSEM INSTN'S NL	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES: ^b	PROGRAM ELEMENT 62760A	PROJECT NUMBER 3A762760A834	TASK AREA NUMBER 02	11. LEVEL OF SUM A. WORK UNIT 416			
c. CONTRIBUTING	c. CONTRIBUTING CARDS 114(e)(f)						
12. TITLE (Pecede with Security Classification Code) (U) Mechanisms of immunoprophylaxis against respiratory diseases							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^b 003500 Clinical medicine; 004900 Defense; 010100 Microbiology							
13. START DATE 72 08	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house				
17. CONTRACT/GRAANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS			
B. DATES/EFFECTIVE:		EXPIRATION:		FISCAL YEAR	CURRENT 76	1.0	173.8
D. NUMBER: ^b NA		E. AMOUNT:		FISCAL YEAR	CURRENT 77	1.0	156.0
G. TYPE:		F. CUM. AMT.		20. PERFORMING ORGANIZATION			
G. KIND OF AWARD:				NAME: Aerobiology Division USAMRIID ADDRESS: Fort Detrick, MD 21701			
19. RESPONSIBLE DOD ORGANIZATION				PRINCIPAL INVESTIGATOR (Pecede DOD or U.S. Academic Institution) NAME: Jemski, J. V. TELEPHONE: 301 663-7453 SOCIAL SECURITY ACCOUNT NUMBER:			
NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701				ASSOCIATE INVESTIGATORS NAME: POC:DA			
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833							
21. GENERAL USE Foreign intelligence considered							
22. KEYWORDS (Pecede EACH with Security Classification Code) (U) Immunoprophylaxis; (U) BW defense; (U) Military medicine; (U) Respiratory pathogens; (U) Airborne infections; (U) Mycoplasma pneumoniae; (U) Animal models							
23. TECHNICAL OBJECTIVE, ^b 24. APPROACH, 25. PROGRESS (Pecede individual paragraphs identified by number. Pecede each with Security Classification Code.)							
23 (U) Characterize host immunity against respiratory infections by depositing microbial antigen as aerosols or intranasal (IN) instillation in selected regions of the respiratory tract. Determination of optimal methods of respiratory immunization is an essential element in military medicine as it would be a major determinant of resistance against potential respiratory pathogens used in BW.							
24 (U) Using influenza vaccines (killed and attenuated) study the efficacy of different methods of immunization including large and small particle aerosols against a respiratory challenge with virulent virus.							
25 (U) 75 07 - 76 06 - Small-particle aerosols (SPA) of Mycoplasma pneumoniae were more efficient in initiating infection in the hamster than large-particle aerosols (LPA). SPA also are comparable to intranasal instillation of large volumes (200 microliters) of mycoplasma in initiating both upper and lower respiratory tract infection. LPA and small intranasal inocula (20 or 2 microliters) induce a predominantly upper respiratory tract infection. All initially infected hamsters were protected to varying degrees against subsequent challenge with homologous M. pneumoniae. The best protection appeared in the group initially infected with SPA; large volume intranasal was second in effectiveness. These data indicate that optimal immunization against lower respiratory tract diseases probably must include stimulation of the local immune defense mechanism in the lower respiratory tract.							
Publications: Infect. Immunity 13:818-824, 1976							
Available to contractors upon originator's approval.							

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 416: Mechanisms of Immunoprophylaxis Against Airborne Infections

Background:

The significance of a nonviral respiratory disease, designated as primary atypical pneumonia caused by Mycoplasma pneumoniae, has been established during the past decade. Although not a fatal disease, M. pneumoniae is the most important cause of pneumonia in older children and young adults.¹ The disease is a serious problem in the military, as particular high-risk groups are military recruits and college students.² Vaccines and immunization procedures for the prevention of primary atypical pneumonia have been unsuccessful to date. Using the hamster model much critical insight, however, has been provided in vaccine development and on the pathogenesis of respiratory infection due to M. pneumoniae. It has been shown that IN infection of hamsters resulted in superior protection to rechallenge than that for hamsters parenterally injected with either living or inactivated vaccines.³ This suggested that the most effective protection would be provided if the local respiratory immune systems in the respiratory tract could be stimulated. Our investigations, therefore, have been directed toward studying aerogenic immunoprophylaxis against respiratory diseases by exposing hamsters to small- and large-particle aerosols (SPA and LPA) of M. pneumoniae which are deposited selectively in the respiratory tract. The response of hamsters to rechallenge was compared to the response performance of hamsters infected and challenged by the conventional IN route.

Progress:

Various experiments were performed in which groups of 20-30 hamsters were exposed to virulent M. pneumoniae (PI 1428, Pass. 2) aerosols of prescribed sizes or inoculated by the IN route. The research was in collaboration with Dr. R. M. Chanock's group at the Laboratory of Infectious Diseases, NIAID, Bethesda, MD. Anesthetized hamsters were given mycoplasma IN in either 200-, 20-, or 2- μ l volumes. Unanesthetized hamsters were exposed for 10 min to SPA (2.3 μ m) or to LPA (>8 μ m) of M. pneumoniae. Controls included hamsters inoculated IN with sterile broth or exposed to sterile broth aerosols. Immediately after exposure and at predetermined intervals thereafter, hamsters were anesthetized, blood collected for serology, and lung and nasal turbinates cultured. Representative

lung sections were obtained for histological examination and the presence and severity of disease scored on the basis of 0 to 8. All groups were rechallenged IN with $\sim 10^6$ CFU/ml of M. pneumoniae 4-8 wk after initial infection. Between 10 and 14 days after challenge, hamsters were anesthetized and lungs cultured for recovery of mycoplasma. Lung tissues also were evaluated histologically for pneumonia.

Initial experiments compared the response of hamsters infected with SPA or LPA to the response of hamsters infected with a similar dose by the conventional IN route. Mycoplasma were recovered primarily from the nasal turbinates in the SPA group within 2 hr after exposure. At 1 and 2 wk all lungs and nasal turbinates yielded positive cultures. By 3 wk and continuing through 6 wk, organisms were recovered only from the lungs. Organisms were isolated from only a small percentage (10-15%) of the LPA group at 1-4 wk following exposure and then only from the nasal turbinates, indicating an upper respiratory tract infection. The pattern of recovery of organisms from the group receiving a 200- μ l volume IN was similar to that of the SPA group, with involvement of both the upper and lower respiratory tracts. High levels of mycoplasma (10^4 - 10^6 CFU/gm of lung) were observed in the SPA group 1-6 wk after exposure and 1-4 wk for the IN hamsters, while the LPA group had essentially no detectable organisms. These results, as far as can be determined, are the first to describe M. pneumoniae infection by experimental aerosol. These data also strongly support epidemiological studies which have implicated aerosol particles as the primary mode of transmission of M. pneumoniae infection.⁴

Lung lesions and serum CF antibody data from the various infected groups are shown in Table I. Control animals showed no evidence of pneumonia or serum CF antibody 2 wk postinoculation. In contrast, 100% of

TABLE I. RESPONSE OF HAMSTERS 14 DAYS POSTEXPOSURE TO DIFFERENT MODES OF EXPOSURE TO M. PNEUMONIAE

GROUP	DOSE, \log_{10} CFU	N	% WITH PNEUMONIA (lesion score ≥ 3)	LUNG Lesion Score Mean \pm SEM	GEOM. MEAN CF TITER, \log_2 \pm SEM
Control	-	37	0	0.1 \pm 0.1	1.3 \pm 0.1
IN (200 μ l)	6.3	13	100	4.8 \pm 0.5	3.5 \pm 0.3 ^a
	4.4	10	20	0.9 \pm 0.4	1.0 \pm 0.0
	3.0	9	56	2.2 \pm 0.7	1.1 \pm 0.1
SPA	4.6	14	36	1.9 \pm 0.4	1.4 \pm 0.3
LPA	4.2	19	0	0.1 \pm 0.1	1.4 \pm 0.2

^aP < 0.001, Student's t test.

the animals inoculated IN with a high dose of $10^{6.3}$ CFU/200 μ l developed severe pneumonia. Lower doses (10^3 - $10^{4.4}$ CFU) presented IN in the same size volume produced less severe pneumonia in 20-56% of the animals. Like the lower dose IN groups, a smaller percentage (36%) of SPA-treated hamsters which had received $10^{4.6}$ CFU demonstrated low grade pneumonia. Hamsters inhaling $10^{4.2}$ CFU presented as LPA did not develop pneumonia.

Since animals aspirating 200 μ l IN developed infections of both upper and lower respiratory tract, hamsters received smaller IN volumes as a possible means of restricting infection to the upper respiratory tract. Four logs of mycoplasma were administered IN in volumes of 200-, 20-, and 2- μ l. Both lungs and nasal turbinates were infected with the 200- μ l volume. In contrast, mycoplasma were recovered only from the nasal turbinates of the hamsters receiving the two smaller inocula for up to 4 wk after infection. Lung cultures from these animals were consistently negative.

LPA-, SPA- and IN-infected hamsters, as well as broth control groups, were challenged 4-8 wk after initial exposure to determine whether protective immunity had developed. Challenge consisted of 10^6 CFU/ml of M. pneumoniae administered IN in a 200- μ l volume to assure delivery to the lower respiratory tract. The results are summarized in Table II.

Of the control animals, 96% developed pneumonia 10 days after challenge. The mean lung lesion score of the control group was 5.3; ~6 logs of organisms were present in the lungs. All groups previously exposed to M. pneumoniae, whether by aerosols or IN administration, showed significantly reduced pulmonary disease as reflected by incidence of pneumonia and lung lesion scores.

Prior exposure to M. pneumoniae in SPA (which infect both upper and lower respiratory tracts) provided the greatest protection against reinfection with only a 11% incidence of pneumonia observed and a mean lung lesion score of <1.0. Significantly fewer challenge organisms were found in the lungs of animals previously exposed to SPA than in the control group. In contrast, the LPA-treated group in which the previous infection was limited largely to the upper respiratory tract, had a 60% incidence of pneumonia with a mean lung lesion score of 2.9. Although the frequency of pneumonia was significantly lower in the LPA group relative to controls, the frequency of pneumonia and mean lung lesion score in the LPA group were significantly higher than the SPA group.

Only 36% of hamsters previously exposed to an IN 200- μ l volume developed pneumonia following challenge. The mean lung lesion score of this group was 1.4; also significantly less than the LPA group. From 42-50% of the animals in the 2 groups that received the low volumes developed pneumonia with a mean lung lesion score of ~2. The 2- μ l group frequency and severity of pneumonia were very similar to the LPA group.

The overall results of these studies showed that varying levels of protection against pneumonia were obtained in hamsters following challenge

TABLE II. EFFECT OF PRIOR M. PNEUMONIAE EXPOSURE ON HAMSTERS CHALLENGED INTRANASALLY WITH M. PNEUMONIAE (10^6 CFU) 10 DAYS POSTEXPOSURE

GROUP	DOSE, \log_{10} CFU	N	% WITH PNEUMONIA (lesion score >3)	LUNG LESION SCORE, MEAN \pm SEM	\log_{10} CFU/GM LUNG, MEAN \pm SEM
Control	-	58	96	5.3 ± 0.3	5.7 ± 0.2
SPA	4.6	18	11 ^a	0.9 ± 0.2^c	3.4 ± 0.5^e
LPA	4.2	30	60 ^a	2.9 ± 0.5^c	4.2 ± 0.4
IN				c	d
200 μ l	4.4	19	36 ^a	1.4 ± 0.3^c	4.6 ± 0.2
20 μ l	4.0	19	42 ^a	1.6 ± 0.5^c	4.8 ± 0.3
2 μ l	3.8	18	50 ^a	2.1 ± 0.4^c	5.1 ± 0.4

^aP < 0.001, vs. controls (Chi square with Yate's correction).

^bP = 0.002, (Fisher's exact test).

^cP < 0.001, vs. controls (Fisher's protected Least Significant Difference).

^dP < 0.005, LPA vs. SPA; P < 0.05, LPA vs. 200 μ l. IN (Fisher's protected Least Significant Difference).

^eP < 0.001, vs. controls (Student's t test).

with virulent M. pneumoniae. The degree of protection appeared dependent on the particle size of the aerosol and on the volume of the IN inoculum used for initial infection of the hamsters. Our studies also showed that protection was best in the group initially infected by SPA based on lung lesion scores, the low percentage of animals developing pneumonia after challenge and very importantly, the significant suppression of the challenge organisms in the lung. This indicates that optimal immunization against M. pneumoniae probably must include antigenic stimulation of the local immune defense mechanism in the lower respiratory tract.

The potential of the Mongolian gerbil as an alternate model to the widely used hamster for experimental M. pneumoniae infections was investigated. Following IN administration of M. pneumoniae, the response of the gerbil paralleled that of the hamster, particularly in that no overt illness was discernible. Hematological parameters (total and differential WBC and hematocrit) were not altered by the infection (similar to the hamster). The peribronchiolitis seen in the gerbil lung 14 days after infection was

comparable to that obtained in the hamster. Experiments also have been initiated on the squirrel monkey as another model for M. pneumoniae.

Other major efforts undertaken were in collaboration with MAJ Powanda, Work Unit No. 834 01 401. One was to determine reference data on blood lipid, protein and trace metal concentrations of hamsters at various intervals following initial infection with M. pneumoniae and after rechallenge. Secondly, the effect of a 2% clofibrate diet, an antilipemic agent, on the cholesterol levels of infected and noninfected hamsters was to be evaluated. As rodents, hamsters have unusually high plasma cholesterol levels and cholesterol is an essential metabolite for M. pneumoniae. Data accumulated so far indicate that despite a 50% lowering of the cholesterol level in hamsters ingesting clofibrate, the response of these animals remained essentially the same as infected hamsters on a normal diet. It appears, therefore, that high concentrations of clofibrate fed to hamsters does not influence the infectivity and pathogenicity of the virulent M. pneumoniae administered to these animals.

Efforts are continuing to positively identify presumptive microcolonies of M. pneumoniae in the bronchi and alveoli of experimentally infected hamsters by interaction with antibody-sensitized beads (in collaboration with Dr. John White, Work Unit No. 834 01 406). Equivocal results were obtained with beads sensitized with antibody prepared against the whole organisms. Attempts are being made to obtain more highly titrated antibody as well as antibody directed against the lipid antigens of M. pneumoniae.

Presentation:

Hetsko, C., J. Jemski, C. Helms, M. Grizzard, J. Walker, and R. Chanock. Experimental infection of hamsters with aerosols of Mycoplasma pneumoniae (MPn). Presented, 15th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, D.C., 24-27 Sep 1975 (Abstracts, No. 289).

Publications:

1. Jemski, J. V., and J. S. Walker. 1976. Aerosol vaccination of mice with a live, temperature-sensitive recombinant influenza virus. *Infect. Immun.* 13:818-824.
2. Jemski, J. V., C. M. Hetsko, C. M. Helms, M. B. Grizzard, J. S. Walker, and R. M. Chanock. 1976. Immunoprophylaxis of experimental Mycoplasma pneumoniae disease. II. Effect of aerosols of M. pneumoniae on the induction of respiratory infection, disease and immunity in hamsters. *Infect. Immun.* 14, in press, 1976.

LITERATURE CITED

1. Chanock, R. M. 1965. Mycoplasma infections of man. *N. Engl. J. Med.* 273:1199-1206.

2. Sternberg, P., R. J. White, S. L. Fuld, R. R. Gutekunst, R. M. Chanock, and L. B. Senterfit. 1969. Ecology of Mycoplasma pneumoniae infections in Marine recruits at Parris Island, South Carolina. Am. J. Epidemiol. 89:62-73.
3. Fernald, G. W., and W. A. Clyde, Jr. 1970. Protective effect of vaccines in experimental Mycoplasma pneumoniae disease. Infect. Immun. 1:559-565.
4. Sande, M. A., F. Gadot, and R. P. Wenzel. 1975. Point source epidemic of Mycoplasma pneumoniae infection in a prosthodontics laboratory. Am. Rev. Resp. Dis. 112:213-217.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^b DA OE6412	2. DATE OF SUMMARY ^b 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUMRY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SECY ^b U	6. WORK SECURITY ^b U	7. REGRADING ^b NA	8. DA DISGPN INSTR'N NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: ^b PROGRAM ELEMENT		PROJECT NUMBER		11. TASK AREA NUMBER 02	12. LEVEL OF SUM A. WORK UNIT 417	
a. PRIMARY 62760A	3A762760A834					
b. CONTRIBUTING						
c. CONFIDENTIAL CARDS 114(e)(f)						
11. TITLE (Provide each with Security Classification Code) (U) Basic properties and clinical application studies on transfer factor						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^b 003500 Clinical medicine; 004900 Defense; 010100 Microbiology						
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29. RESPONSIBLE DOD ORGANIZATION		30. PERFORMING ORGANIZATION		31. PRINCIPAL INVESTIGATOR (Provide name if U.S. Academic Institution) NAME: Ascher, M. S. TELEPHONE: 301 663-7341 SOCIAL SECURITY ACCOUNT NUMBER:		
32. RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		33. ASSOCIATE INVESTIGATORS NAME: Andron, L. A.		34. POC:DA		
35. KEY WORDS (Provide each with Security Classification Code) (U) BW defense; (U) Military medicine; (U) Cell-mediated immunity; (U) Infectious diseases; (U) Lymphocyte transformation; (U) Transfer factor						
36. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Punish individual paragraphs identified by number. Provide each with Security Classification Code.) 23 (U) Study cell-mediated immunity (CMI) by lymphocyte transformation to microbial antigens in man and experimental animals in model infectious diseases. Assess the role of transfer factor (TF) as an immunostimulant or a specific prophylactic in infections of military medical significance and potential BW threat. 24 (U) Develop a microtechnique for assay of transfer factor using lymphocyte transformation of cells from monkeys and humans known to be sensitized to tularemia, Venezuelan equine encephalomyelitis, Q fever, or Rocky Mountain spotted fever. Transfer factor will be prepared from the same donors. Various biochemical techniques will be utilized as appropriate. 25 (U) 75 07 - 76 06 - Using the in vitro lymphocyte transformation assay developed in this laboratory, studies have continued on the nature and mode of action of dialysates containing TF. It has been established that the concept of donor specific transfer, long a feature of in vivo work, cannot be shown in our in vitro system. In other words, active leukocyte dialysate which enhances lymphocyte proliferation to specific antigen can be derived equally well from individuals with or without exposure to the test antigen. In addition, such material can act late in culture, appears to pass through a 1,000 molecular weight membrane filter, and can be derived from diverse tissue culture sources. The immunopotentiating effects of these products is under intensive investigation for use in the prophylaxis of infectious diseases in man and experimental animals. Publications: Fed. Proc. 35:337 (2 abstracts), 1976. Cell. Immunol. 23:32-38, 1976. In Transfer Factor -- Basic Properties and Clinical Applications, p. 3-12, 291-300, 1976.						
37. Available to contractors upon contractor's approval.						

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PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

* U.S. GPO: 1974-840-843/8891

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 417: Basic Properties and Clinical Application Studies on Transfer Factor

Background:

Recognition of the importance of cell-mediated immunity (CMI) in host resistance to a variety of infectious diseases has motivated interest in both naturally occurring and synthetic reagents which can be used to reconstitute or modulate the cellular immune response in man. Because dialysable transfer factor (TF) is nontoxic, nonimmunogenic, and free of infectious material, Lawrence has proposed it to be the substance of choice for inducing CMI in humans.^{1,2} Clinical trials with TF demonstrated variable success in treatment of fungal, bacterial, viral and malignant diseases, but development of an improved mode of therapy was hindered by the lack of a successful animal model system or a reliable in vitro assay for TF activity. Employing a lymphocyte microassay described previously^{3,4} we have been able to define some fundamental physical characteristics of an activity in TF that enhances or augments lymphocyte responses to antigen in vitro. Although the basic chemical nature of TF is still unknown, this project concerns development of methods for measurement of CMI in man and experimental animals and for testing for effects of TF on such responses in vivo and in vitro.

Progress:

To establish an animal model system in which to test the immuno-potentiating effects of TF, we developed and characterized an efficient whole blood in vitro lymphocyte transformation technique for guinea pigs. Heparinized blood diluted 10-fold in RPMI-1640 was incubated in the presence of antigen for 5 days and cellular proliferation was assessed by standard thymidine incorporation techniques. The data presented in Table I indicate that immunization with killed tularemia organisms produced a slight increase in lymphocyte transformation to specific antigen, whereas vaccination with the tularemia living vaccine strain produced a dramatic increase.

TABLE I. IN VITRO LYMPHOCYTE TRANSFORMATION RESPONSE (INCORPORATION OF [^{14}C]THYMIDINE) OF GUINEA PIG LYMPHOCYTES TO TULAREMIA ANTIGEN.

IMMUNIZING ANTIGEN	CPM OF LYMPHOCYTE CULTURES BY DAY AFTER IMMUNIZATION						
	1	14	27	48	52	63	129
None	52	57	124	112	71	66	59
Killed	89	102	268	234	181	89	67
Living vaccine strain	99	168	1182	1857	260	125	218

Studies are in progress to assess the potential enhancing effects of TF and a variety of synthetic adjuvant materials such as Levamisole and Poly I:C in this system. Collaborative efforts with the Rickettsiology Division (Work Units 834 02 300 and 834 02 303) resulted in establishing a similar test system with rickettsial antigens in guinea pigs. In addition, studies in collaboration with the Medical Division have involved testing recipients of the new RMSF vaccine for lymphocyte transformation with RMSF antigen. In collaboration with MAJ Anderson (Work Unit 834 02 013) effects of TF have been shown on lymph node morphology and cellular traffic in a rat model. In addition, LTC Houston (Work Unit 834 02 419) has demonstrated an immuno-potentiating effect of TF on antibody formation to a killed viral antigen. These projects are described elsewhere in this report.

One of the most challenging concepts about TF in vivo is the principle of donor specific transfer. Many studies have been reported indicating in general that those sensitivities possessed by the TF donor appear in the recipient after transfer. However, extraneous sensitivities have occasionally appeared in patients receiving TF.² To test the in vitro specificity of dialysates containing TF, a series of experiments were performed using donors of well-defined reactivity to the following antigens: streptokinase-streptodornase (SK-SD) and an antigen derived from the RMSF vaccine. Eight individuals were screened by lymphocyte transformation to these 2 antigens, and 4, representing the 4 combinations of positive and negative responses, were selected for preparation of TF from their leukocytes. The respective dialysates were assayed simultaneously with both antigens in cultures of recipient lymphocytes from a single individual (Table II). A high level of enhancement of recipient lymphocyte proliferation was engendered by TF preparations obtained from either high or low responding donors.

TABLE II. COMPARISON OF SENSITIVITY OF TF DONORS WITH ABILITY OF THEIR TF TO AUGMENT IN VITRO RESPONSIVENESS ($[^{14}\text{C}]$ THYMIDINE INCORPORATION) OF RECIPIENT LYMPHOCYTES FROM A SINGLE INDIVIDUAL.

DONOR TF PREPARATION	ANTIGEN ADDED	Donors	CPM FOR LYMPHOCYTES FROM			
			Recipients			
			Dose of TF _{DM} (μl) ^a	0	50	100
1	SK-SD	4016	2054	3504	4073	4529
	RMSF	4996	252	320	1217	1700
2	SK-SD	823	2054	3550	4197	4868
	RMSF	8457	252	381	827	1903
3	SK-SD	6980	2054	3846	4160	4386
	RMSF	256	252	309	672	1981
4	SK-SD	386	2054	4130	4350	4702
	RMSF	279	252	346	768	2024

^a TF_{DM} = Transfer factor, dialyzed and sterilized by Millipore filtration.

Several considerations were required to reconcile these results with the in vivo effects of TF. (1) The effects were measured on cells that are already responding to antigen; we have been unable to affect cells completely unresponsive to antigen. Thus, it is possible that initiation of in vivo activity requires a specific factor in addition to the nonspecific enhancement factor(s). (2) This material could facilitate expansion of a memory clone and other materials responsible for skin test transfers are indeed antigen specific. (3) Nonspecific effects of TF have been demonstrated in vivo and the in vitro transformation enhancement factor could be responsible for these changes; the relative contribution of such nonspecific effects to patient responses in clinical situations has yet to be determined. (4) If in vivo experiments could be performed with adequate reciprocal controls it is conceivable that the ability to transfer is less a function of donor sensitivity than a combination of recipient sensitivity and relative antigen potency. None of these possibilities are excluded by the data currently available; therefore, clarification will require careful design of both in vivo and in vitro experiments.

Using our lymphocyte transformation microassay, studies have been initiated to determine the basic physical properties of components responsible for enhancing *in vitro* activity. We have shown that filtrates with activity were recovered after passage through a Millipore membrane with a nominal molecular weight cutoff of 1000; some activity, however, still remained in the retentate.

The effect of delayed additions of 50, 100, or 150 μ l of TF dialysate on [^{14}C]thymidine incorporation by human lymphocytes incubated with SK-SD antigen was examined (Table III). Despite delayed addition, all doses enhanced lymphocyte proliferation. The 50- μ l dose of active material added as late as day 3 yielded enhancement equivalent to that for the same dose added on day 0, 1 or 2. The effect of higher doses, however, was somewhat decreased when TF was added after day 2. These data suggest that the dialysate's effect is on cell proliferation per se rather than on antigen recognition, an early event of transformation.

TABLE III. EFFECT OF DELAYED ADDITION OF 50, 100 OR 150 μ l OF TF DIALYSATE ON [^{14}C]THYMIDINE INCORPORATION BY HUMAN LYMPHOCYTES INCUBATED WITH SK-SK ANTIGEN FOR 7 DAYS.

DOSE OF TF (μ l)	CPM FOR CULTURES WITH TF ADDED BY DAYS			
	0	1	2	3
0	2397	2517	2182	2217
50	5971	7654	6447	5633
100	8576	6310	6161	4825
150	7766	7135	6040	4458

Additional support for the nonspecific nature of *in vitro* activity in TF dialysates was engendered by reports that TF-like responses could be induced by dialysates prepared from cells other than those of hematopoietic origin.⁵ Therefore, dialysates were derived from WI-38 fibroblasts, mouse L-cells and baby hamster kidney cells. Human lymphocytes from one individual were cultured with tuberculin PPD and increasing doses (50-150 μ l) of each dialysate in the manner described previously. The results (Table IV) confirm that *in vitro* TF-like activity can be induced by dialysates prepared from diverse cell lines and is not restricted to cells in the hematopoietic systems. Preliminary results of chromatographic separation of such extracts indicates that their activity profiles differ from those described for material of leukocytic origin; this distinction will be pursued further.

TABLE IV. ENHANCEMENT OF [^{14}C]THYMIDINE INCORPORATION BY HUMAN LYMPHOCYTES CULTURED WITH TUBERCULIN PPD AND DIALYSATES PREPARED FROM TISSUE CULTURE CELL LINES.

SOURCE OF DIALYSATE	CPM FOR LYMPHOCYTES CULTURED WITH DIALYSATE (μl)			
	0	50	100	150
WI-38 fibroblasts	139	ND ^a	656	895
Mouse L-cells	499	1193	1374	1364
Baby hamster kidney cells	219	978	1116	1635

^a ND = not done.

The principal investigators of this project served as hosts for an International Workshop of Basic Properties and Clinical Applications of Transfer Factor in October, 1975. Over 150 scientists from 8 nations met to present 70 formal papers and participate in discussions on recent progress in TF work. The proceedings of this workshop will be published this year by Academic Press.⁵

Presentations:

- Ascher, M. S., and L. A. Andron. Transfer factor (TF) in vitro: nonspecificity of leukocyte dialysates that enhance lymphocyte proliferation to antigen. Presented, Annual Meeting, FASEB, Anaheim, CA., 12-16 April 1976. (Fed. Proc. 35:337, 1976).
- Andron, II, L. A., and M. S. Ascher. Transfer factor in vitro: chromatography of components that enhance antigen induced lymphocyte transformation. Presented, Annual Meeting, FASEB, Anaheim, CA., 12-16 April 1976. (Fed. Proc. 35:337, 1976).

Publications:

- Marker, S. C., and M. S. Ascher. 1976. Specific in vitro lymphocyte transformation with Venezuelan equine encephalitis virus. Cell. Immunol. 23: 32-38.

- 329
2. Ascher, M. S., and L. A. Andron. 1976. In vitro properties of leukocyte dialysates containing transfer factor: micro method and recent findings, p. 3-12. In Transfer Factor: Basic Properties and Clinical Applications, (M. S. Ascher, A. A. Gottlieb, and C. H. Kirkpatrick, ed.), Academic Press, New York.
3. Andron, L. A., and M. S. Ascher. 1976. Chromatography of transfer factor (TF) and assay of fractions in vitro, p. 291-300. In Transfer Factor: Basic Properties and Clinical Applications, (M. S. Ascher, A. A. Gottlieb, and C. H. Kirkpatrick, ed.), Academic Press, New York.
4. Ascher, M. S., A. A. Gottlieb, and C. H. Kirkpatrick (eds.). 1976. Transfer Factor: Basic Properties and Clinical Applications. Academic Press, New York.

LITERATURE CITED

1. Lawrence, H. S. 1969. Transfer Factor. *Adv. Immunol.* 11:195-266.
2. Lawrence, H. S. 1974. Transfer Factor in Cellular Immunity. *Harvey Lecture Series*, 68:239-350, Academic Press, New York.
3. Ascher, M. S., W. J. Schneider, F. T. Valentine, and H. S. Lawrence. 1974. In vitro properties of leukocyte dialysates containing transfer factor. *Proc. Nat. Acad. Sci.* 71:1178-1182.
4. Ascher, M. S., and L. A. Andron. 1975. In vitro properties of leukocyte dialysates containing transfer factor: micro method and recent findings. *Clin. Res.* 23:287A.
5. Ascher, M. S., A. A. Gottlieb, and C. Kirkpatrick (eds.). 1976. Transfer Factor: Basic Properties and Clinical Applications. Academic Press, New York.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ³ DA OE6416	2. DATE OF SUMMARY ⁴ 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUMRY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCRTY ⁵ U	6. WORK SECURITY ⁶ U	7. REGRADING ⁷ NA	8. DISP'N INSTR'N NL	9. SPECIFIC DATA-CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: ⁸ a. PRIMARY 62760A		PROGRAM ELEMENT PROJECT NUMBER 3A762760A834		TASK AREA NUMBER 02		9. LEVEL OF SUM A. WORK UNIT 419
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25. KEYWORDS (Proceed EACH with Security Classification Code) (U) Stabilized poly I:C (PICLC); (U) Encephalitis, equine (VEE); (U) Military medicine; (U) BW defense; (U) Immune enhancement; (U) Adjuvant; (U) Vaccine; (U) Monkeys 26. TECHNICAL OBJECTIVE, ²⁴ APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number. Proceed last of each with Security Classification Code.) 23(U) Inactivated vaccines have been developed to control infectious diseases; they often have marginal potency, and cannot be used to stop a disease outbreak or in a BW situation. Adjuvants plus vaccine and immune complexes frequently evoke more rapid and prolonged protective immunity. This work unit will develop new methods to enhance the immunogenicity of available, marginally antigenic, inactivated viral vaccines for military personnel. 24 (U) A complex at equivalence is used to immunize monkeys and mice. Antibody production and protection against challenge are determined. Adjuvant effects are measured. 25 (U) 75 07 - 76 06 - Enhancement of humoral immune response of monkeys and protection in mice for formalin-inactivated vaccine was shown, if vaccine was complexed at equivalence with specific IgG. IgG antibody was elicited to complexes at equivalence. Adjuvant effects of DEAE-dextran and PICLC were seen in laboratory animals immunized with IVEE vaccine. PICLC stimulated IVEE antibody responses in monkeys to levels similar to TC-83 VEE, and persisted 2.5 mon. PICLC must be combined with IVEE rather than given separately. Dose responses showed that potentiation of the IVEE antibody is elicited with a single dose of 10 micrograms/kg. Immune enhancement was seen in monkeys immunized with JE-IgG complexes at equivalence. There were higher neutralizing antibody titers, detectable earlier and persisting longer than controls. Course of infection and lethality were established for 2 monkey species, following IN challenge with JE virus; a monkey IN lethal dose was determined. A lethal primate model was characterized for determining immunogenicity and protection afforded monkeys by complexed group B viruses Publication: Am. Soc. Microbiol. Abstracts, 259, 260, 1976; Arthropod-Borne Virus Information Exchange 30:141-142, 1976; Infect. Immunity 13:1559-1562, 14:318-319, 1976.						
*Available to contractors upon originator's approval.						

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BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 419: Effect of Complexed Antigen on the Immune Response of Nonhuman Primates to Vaccine.

Background:

Part I. Formalin-inactivation of virus vaccines significantly reduces host toxicity that is often associated with administration of live, attenuated vaccines. Many times this process also decreases the antigenicity and protective effects of the vaccine. To compensate for this, various methods including the use of adjuvants have been used to potentiate immunogenicity.

To date, 2 potential adjuvants have been studied, DEAE-dextran, and lysine stabilized polyriboinosinic-polyribocytidyllic acid (PICLC). Complexes of polyinosinic-polycytidyllic (poly I·poly C) acid are effective interferon inducers in rabbits and mice;¹ however, it is a weak inducer of interferon in man and nonhuman primates,² and only weakly potentiates the antibody response of monkeys to influenza virus vaccine.³ A recent procedure to stabilize poly I·poly C has resulted in enhanced interferon production in nonhuman primates.⁴

Part II. Immune enhancement in rhesus monkeys and protection in mice have been reported following immunization with formalin-inactivated Venezuelan equine encephalomyelitis (IVEE) vaccine complexed with specific immune γ -globulin (IgG) at equivalence.⁵ If enhancement of the primary humoral immune response can be demonstrated in monkeys following immunization with JE virus antigen-antibody complexes, it may be possible to achieve in man more rapid and persistent humoral antibody response to inactivated group B arbovirus vaccines having weak immunogenic properties.

Part III. Working with a bacterial product complex (bacterial α -amylase and specific antibody), Osato⁶ established that the enhancement of the Ab response by Ag-Ag complexes was dose-dependent and affected by the route chosen for immunization. The greater immunogenicity of the Ag-Ab complex, in comparison with free Ag, was investigated by *in vitro* and *in vivo* vascular clearance studies as to whether a quantitative difference in the rate of uptake by phagocytes was a significant factor in the Ab response. If a difference in the rate of phagocytosis, particularly by macrophages which are important for the induction of Ab synthesis, and degradation does exist, it may be responsible for the greater Ab enhancement by the complex.

The degraded products of phagocytized complexes were highly immunogenic fragments capable of eliciting enhanced primary and secondary Ab responses as shown by in vitro and in vivo clearance studies.⁶ However, this was in contrast to the degraded products of phagocytized Ag which did not elicit primary or secondary Ab responses. Therefore, a qualitative difference, i.e., a different mechanism or route, in the manner of degradation by phagocytes for complexes and free Ag was suggested.

The degraded antigenic fragments of phagocytized complexes (bacterial α -amylase-specific Ab) showed greater immunogenicity than the original complex in obtaining a primary Ab response.⁶ In addition, a greater secondary Ab response from primed cells was elicited by the degraded products of the phagocytized complex than both the original complex or free Ag.

Progress:

Part I. The adjuvant effect of DEAE-dextran (DEAE-D) was presented last year. In brief, significant potentiation of the humoral immune response of rhesus monkeys to IVEE was demonstrated with DEAE-D administered in concentrations of 1 and 5 mg/kg in combination with IVEE. This year we report further characterization of this response in terms of class of antibody elicited. Table I shows the plaque reduction neutralizing (PRN) titers of 2 groups of 4 monkeys each; 1 group administered SC IVEE alone, and another group given IVEE plus 5 mg/kg DEAE-D by the same route. A potentiation of the immune response by DEAE-D is evident in whole serum titers. In an attempt to define differences in the 2 immune responses, the immunoglobulin class of antibody that developed following vaccination was determined. The PRN antibody responses following administration of both vaccine preparations was biphasic. Although titers of IgG antibody peaked at the same time (day 18) in response to both VEE antigens, specific IgG antibody elicited by the vaccine plus adjuvant reached a substantial titer by day 10. In contrast, the neutralizing capacity of IgG antibody was not detected until day 14, and was not the major contributor until day 18 in monkeys given vaccine alone. The significance of the more rapid IgG response to the use of DEAE-D as an effective adjuvant is discussed in a recent manuscript.¹

A considerable effort during the last year has been spent in evaluating PICLC for its potential as an effective adjuvant when used in conjunction with IVEE. PICLC was diluted in distilled water and combined with IVEE to inoculate each monkey with 0.5 ml or rats and mice with 0.3 ml of the vaccine and either 3, 1, 0.5, 0.1 or 0.01 mg/kg body weight. The adjuvant effect of PICLC was first examined in Lewis rats. First, the humoral response of the rats to IVEE and PICLC was determined. IVEE was inoculated SC in the vicinity of the axillary lymph node, and PICLC was injected also into the area of the axillary node with the antigen, or in the vicinity of the contralateral inguinal lymph node. The response, as measured by PRN antibody titers, are presented in Table II.

TABLE I. PRN ANTIBODY RESPONSES, BY CLASS, OF RHESUS MONKEYS (N=8) IMMUNIZED WITH IVEE AND IVEE + DEAE-DEXTRAN.

DAY	GEOMETRIC MEAN PRN TITER					
	IVEE		IVEE + DEAE-D (5 mg/kg)			
	Whole ^a	IgM	IgG	Whole ^a	IgM	IgG
3	0	0	0	7	0	0
5	8	0	0	64*	10	0
7	27	6	0	152*	28	0
10	45	14	0	430*	34	20
14	38	11	8	362*	27	57
18	38	5	16	304*	0	95
23	16	0	11	430*	0	80
32	8	0	0	181*	0	40
50	8	0	0	215*	0	47
72	0	0	0	215*	0	67

^aWhole serum

* P < 0.01

TABLE II. ADJUVANT EFFECT OF PICLC IN LEWIS RATS (N=4) IMMUNIZED WITH IVEE.

VACCINE	PICLC		PRN TITERS BY DAYS				
	Axillary Node	Inguinal Node	7	10	14	24	35
+	-	-	107	430	22	107	1217
+	1 mg	-	2048*	4096*	1217*	1448*	16,384*
+	-	1 mg	608	304	86	304	1217
+	3 mg	-	861*	1625*	256	4096*	16,384*
+	-	3 mg	430	362	64*	215	430

* P < 0.025

In comparison with IVEE controls the groups of rats inoculated with IVEE in combination with both concentrations of adjuvant (axillary lymph node) responded with significantly higher antibody titers. Apparently, PICLC must be given in combination with IVEE, since little or no potentiation of the immune response was observed if PICLC was inoculated in the contralateral inguinal node to that of antigen. The only enhanced response documented on day 14 in the group given 3 mg/kg PICLC in the inguinal node could be due to the wide range of variability in the antigen control, as compared with consistent titers in the antigen plus PICLC group. All rats in each group were challenged with a lethal dose (10^3 MICLD₅₀) of Trinidad strain VEE on day 35; all were protected.

Due to the strong antibody response and complete protection of Lewis rats to IVEE alone, this animal did not appear to offer a good model for studying adjuvant enhancement of antibody production and its correlation with protection. Although the rat continues to be the animal of choice for studies in the cellular aspects, efforts turned to the mouse, to study (1) adjuvanticity of PICLC for IVEE, (2) antibody production and correlation with protection, and (3) dose responses using graded amounts of the PICLC.

The dose response of PICLC is presented in Table III. PRN antibody and protection are presented for mice immunized with a marginal, 1:10, concentration of IVEE, and graded doses of PICLC. A potentiation in antibody response is obvious, and closely parallels the degree of protection measured at 35 days postvaccination. Eighty-five to 100% protection was recorded with all concentrations of PICLC, even 10 μ g/kg, given only once. This is in contrast to 45% protection and a poor antibody response in antigen controls.

Since the stabilization process that produced PICLC was designed to develop a polynucleotide that would be active in nonhuman primates, we next tested the dose response in rhesus monkeys. The experimental design was identical to that used in mice. Undiluted IVEE was used in the monkeys, since past experience has shown that IVEE diluted 1:10 is not sufficient to produce repeated seroconversion in monkeys. A similar dose-response pattern of antibody production is seen in Table IV. Data are complete through day 28, and a potentiating effect is seen with as little as 10 μ g/kg. With concentrations as small as 1 mg/kg the enhancement is extended for at least 2½ mon, whereas antibody responses in antigen controls are essentially base-line by 28 days. Studies are in progress to determine the toxic effects of PICLC in rhesus monkeys; results are reported in Work Unit 834 02 411.

TABLE III. DOSE-RESPONSE OF PICLC + IVEE WITH RESPECT TO IMMUNE RESPONSE AND PROTECTION OF MICE.

DAY	PRN TITERS (N=10)					
	IVEE + PICLC (mg/kg)					
	3	1	0.5	0.1	0.01	0 (controls)
3	32	32	4	4	4	4
7	64	64	8	6	5	4
10	64	256	9	7	7	5
14	256*	512*	32*	16*	16*	5
21	1024*	1024*				
28	512*	512*	70*	28*	11	6
35	256*	128*	32*	70*	19	6

Protection Study

<u>Survivors</u>	18/19	18/20	20/20	18/20	17/20	9/20
Total						
%	95	90	100	90	85	45

* P < 0.01

TABLE IV. DOSE-RESPONSE OF PICLC ON THE IMMUNE RESPONSE OF RHESUS MONKEYS TO IVEE.

DAY	PRN TITER (N=4)					
	IVEE + PICLC (mg/kg)					
	3	1	0.5	0.1	0.01	0 (controls)
3	4	9	4	4	4	6
7	128	256	14	38	16	54
10	861*	600*	128*	108*	64	76
14	2400*	1200*	215*	128*	45	76
28	2400*	1400*	362*	64*	45*	7
50	2400*	2400*				6
72	600*	304*				6

* P < 0.01

To study further the mechanism of protection mediated by PICLC, 3 groups of 140 mice each were inoculated SC with IVEE (0.3 ml), IVEE + PICLC (3 mg/kg), and PICLC alone. Ten mice from each group were challenged with 5×10^3 MICLD₅₀ Trinidad VEE daily for 14 days and observed for a 21-day period, at the end of which the percentage of protection was measured (Table V).

TABLE V. TEMPORAL PROTECTION^a AFFORDED MICE BY IVEE AND PICLC (3 mg/kg).

DAY	% PROTECTION			
	IVEE + PICLC (n=10)	IVEE Controls (n=10)	PICLC Controls (n=10)	Challenge Controls (n=6)
1	50	10	30	0
2	60	20	20	
3	80	40	10	
4	50	30	0	
5	70	40	0	
6	50	40	0	
7	60	40	0	
8	90	100	0	
9	90	80	0	
10	90	40	0	
11	100	60	0	
12	100	50	30	
13	100	40	20	
14	90	70	0	0

^aChallenged daily for 14 days; held 21 days for protection determination.

Fifty percent of the mice inoculated with IVEE plus the PICLC were protected against lethal challenge by 24 hr. The degree of protection gradually increased during the next 7 days and, by day 8 and for the remainder of the experimental period, 90-100% of the mice were protected.

In contrast only 10% of the mice inoculated with IVEE survived challenge at 24 hr. Eight days were required in this antigen-control group before protection at the 80-100% level was conferred; this high degree of protection did not persist throughout the period. Thirty and 20% of the mice inoculated with PICLC survived challenge on days 1 and 2, respectively. This degree of protection probably reflects interferon (IF) induction in the mice (Table VI). These IF levels were obtained in separate groups of mice. Two separate lots (49 and 59) of PICLC (Levy, National Institutes of Health) were tested for their ability to induce IF. Lot 49 induced IF production by 24 hr, and lot 59 as early as 2 hr. The PICLC used in the test was lot 49. It has been shown that 25 IU of IF are protective against an IF-sensitive virus (VEE) (Stephen, personal communication). The protective levels observed on days 12 and 13 in the PICLC control group is interesting. No explanation is offered at this time; however, a similar observation was made (1 Q, FY 76) in that 30% of mice inoculated with PICLC were protected against lethal challenge at 28 days. One question remains when analyzing these data: could the reason for 10-20% protection in antigen controls on days 1 and 2 postinoculation be due to IF induction by IVEE? It is known that live VEE virus induces IF in burros by 24 hr postinoculation (Stephen, personal communication). Studies are now in progress to determine whether live TC-83, and inactivated VEE virus induce IF, and if this could be an early protective mechanism to VEE.

TABLE VI. COMPARISON OF IF LEVELS^a OF 2 LOTS OF PICLC.

IF LOT	GEOM. MEAN IF LEVELS	
	2 hr	24 hr
49	0	135
59	400	180

^aMeasured by 80% plaque inhibition of VSV virus.

Part II. In vitro Ag-Ab equivalence between JE virus immune monkey γ -globulin (IgG) and isotopically ^3H -JE virus was determined by a modification of the radioisotope precipitation (RIP) technique of Gerloff et al.⁷ Equivalence between Sephadex-separated IgG and formalin-inactivated JE (Townsend) antigen was obtained when equal volumes of undiluted, killed JE (IJE) antigen and 1:2 dilution of IgG were combined. To determine if immunological enhancement could be induced in rhesus monkeys following a single immunization with an Ag-Ab complex other than IVEE, a study was conducted with IJE antigen to investigate the primary immune responses of monkeys to a complexed group B arbovirus antigen (Table VII). Geometric mean antibody titers in monkeys immunized with the Ag-Ab

complexed at equivalence were demonstrable by day 5, whereas 10 days were required before detectable levels of antibody were obtained in the antigen control group (Table VII). Low antibody titers were detectable in the group administered complex in antigen excess in low levels only on day 14, whereas the antibody control group developed no detectable levels of PRN antibody. It is therefore apparent that complexing marginal concentrations of inactivated JE or VEE antigen at equivalence with specific IgG provides a mechanism for enhancing the immunogenicity of an otherwise weakly antigenic inactivated viral vaccine.

TABLE VII. JE PRN ANTIBODY RESPONSES OF RHESUS MONKEYS IMMUNIZED WITH IJE AT EQUIVALENCE, IJE-ANTIGEN EXCESS, IJE ALONE, AND ANTIBODY ALONE.

DAYS AFTER IMMUNIZATION	RECIPROCAL GEOMETRIC MEAN PRN ₈₀		ANTIBODY TITERS ^a	
	IJE at equivalence (n=4)	IJE at antigen excess (n=4)	IJE alone (n=4)	Antibody alone (n=2)
0-3	2.5	2.5	2.5	2.5
5	34.0	2.5	2.5	2.5
7	34.0	2.5	2.5	2.5
10	48.0	2.5	3.5	2.5
14	113.0	5.0	10.0	2.5
21	80.0	2.5	5.0	2.5
28	6.0	2.5	2.5	2.5
35	5.0	2.5	2.5	2.5
43	3.5	2.5	2.5	2.5
52	4.2	2.5	2.5	2.5
65	4.2	2.5	2.5	2.5
73	4.2	2.5	2.5	2.5

^aTiters < 1:10 assigned a value of 1:5.

Occasionally, failures of inactivated JE and VEE virus antigen-antibody complexes to initiate an enhanced immune response in monkeys have been observed. These complexes are formed in what was determined by similar in vitro techniques to be in the zone of equivalence. No definitive reason can be given for these occasional failures; however, several possible explanations are presented.

Previous studies with VEE and JE complexes have established the necessity for combining equivalent proportions of antigen and antibody to obtain immune enhancement. Monkeys immunized with VEE or JE complexes, formed in either antigen or antibody excess, developed low levels of VEE or JE virus PRN antibody.

If the immunogenicity of the formalin-inactivated viral antigen used in the preparation of the complex is decreased, one would not expect to obtain an enhanced response following immunization. Lack of sufficient antigenic mass, variations in the inactivation process, etc., may result in failure of immune recognition of the antigen component of the complex. Human vaccine studies conducted in Asia have shown the necessity for administering multiple doses of IJE vaccine to initiate and maintain significant persistent levels of JE serum neutralizing antibody.⁸ Therefore, additional studies are in progress to investigate the immune responses of monkeys to multiple doses of IJE antigen, complexed antigen, and antigen combined with selected potential adjuvants.

Previous studies by Lee et al.⁹ established that large quantities of virulent JE virus inoculated IV or IM failed to produce signs of encephalitis or death in monkeys. They described a satisfactory JE virus vaccine potency test in Taiwan monkeys (Macaca cyclopis) employing the peripheral IN route of virus challenge.

To establish criteria for determining protection afforded monkeys following immunization with IJE antigens and IJE Ag-Ab complexes to peripheral JE virus challenge, the course of infection and lethal response were determined for 2 species of monkeys following IN challenge with the Peking strain of JE virus. Two rhesus (Macaca mulatta) and 2 cynomolgus (Macaca fascicularis) monkeys were inoculated IN with 3×10^7 PFU of JE virus. Clinical signs included fever, depression, anorexia, tremors, paralysis, and coma. Two monkeys died on day 11, 1 on day 12, and 1 was sacrificed when moribund on day 12. No species-related differences in response to challenge were evident.

In a subsequent study, an IN MLD₅₀ equivalent to 2.5×10^4 PFU of the Peking strain of JE virus was determined with 16 cynomolgus monkeys. A graded dose response manifested by clinical signs and mortality was observed (Table VIII). All of the 5 monkeys in the 2 highest virus-dose groups died, whereas only 2 of 6 in the intermediate dose groups, and none of the 5 in the lowest dose groups developed clinical signs of encephalitis or died.

TABLE VIII. DOSE TITRATION OF JE VIRUS IN MONKEYS.

VIRUS DOSE (PFU)	FEBRILE BY DAY	DEAD/TOTAL	DAY OF DEATH
4×10^6	6	2/2	14, 14
4×10^5	7	3/3	15, 15, 17
4×10^4	9	1/3	13
4×10^3	7	1/3	18
4×10^2	-	0/3	
4×10^1	-	0/2	

To investigate cross-protection afforded monkeys by various group B viruses to challenge with JE virus, 3 groups of rhesus monkeys with different, prior, group B immunization and/or challenge histories were studied (Table IX). Group 1 consisted of 4 monkeys previously immunized with a single marginal dose of either JE Ag-Ab complex formed at equivalence or with JE antigen alone, and challenged ~ 180 days later. Group 2 consisted of 4 monkeys previously immunized and/or challenged with either dengue-2 or yellow fever (YF) virus, or both. The survival of monkeys following virulent YF virus challenge was attributed to post-challenge antiviral chemotherapy. Group 3 consisted of 1 monkey previously hyperimmunized with repeated SC inoculations of virulent JE virus. All monkeys were challenged IN with 4×10^5 PFU JE (Peking) virus, previously shown to be equivalent to ~ 16 monkey INMLD₅₀. The 4 monkeys comprising Group 1, and 3 of 4 monkeys comprising Group 2, developed typical signs of encephalitis and died following challenge. Monkey 469 (Group 2), previously challenged with den-2 and YF viruses, and monkey X-93 (Group 3), previously hyperimmunized with virulent JE virus, became febrile for 3 or 4 days postchallenge; however, neither developed clinical signs of encephalitis nor died. The clinical course following challenge was significantly prolonged in monkeys previously immunized and/or challenged with den-2 or YF virus (Group 2). The mean time to death of Groups 2 and 1 was 34 and 13 days, respectively. The day on which fever (> 103 F) was first observed, as well as the duration of febrile responses, was not markedly different between the 2 groups. The most significant difference in clinical responses was the number of days postchallenge during which the animals were paralyzed and unable to stand, prior to death.

TABLE IX. CLINICAL AND IMMUNE RESPONSES OF RHESUS MONKEYS PREVIOUSLY IMMUNIZED OR CHALLENGED WITH GROUP B ARBOVIRUSES FOLLOWING JE VIRUS CHALLENGE.

MONKEY NO.	PRIOR GROUP B ANTIGENS	RECIPROCAL PRNA ^a TITER				NO. DAYS Febrile	NO. DAYS Paralyzed	DAY OF DEATH
		Prechallenge JE	Prechallenge YF	Prior to Death JE	Prior to Death YF			
GROUP 1								
X-938	Control	4	40	5	40	5	2	12
X-910	Control	<4	<10	<5	10	4	5	16
X-952	Ag alone	<4	<10	20	<10	5	4	13
X-907	Ag alone	<4	<10	20	<10	6	3	12
GROUP 2								
X-394	Den-2 vaccine and challenge + YF challenge	>640	160	2560	6	18		30
X-400	Den-2 vaccine and challenge + YF challenge	20	160	320	1280	5	24	38
469 ^a	Den-2 + YF challenges	40	320	80 ^b	5120	4	0	
545	YF challenge	5	>640	80	5120	8	20	35
GROUP 3								
X-936	JE-hyperimmune	320	10	320	<80	3	0	

^a Survived challenge without developing any clinical signs of encephalitis.

^b JE and YF serum neutralizing (SN) antibody titers 35-42 days postchallenge.

From this initial study, one can not establish minimal protective levels of den-2, YF, or JE SN antibody levels against a moderately large IN challenge dose of JE virus. However, the fact that mean time to death was greatly prolonged in monkeys previously immunized with den-2 and YF viruses, and that 2 of 9 monkeys having different histories of prior group B exposures survived challenge, warrants additional studies to investigate cross-protection conferred by group B arboviruses.

Part III. Using mice as an animal model, the first objective was to determine whether the murine Ab response was enhanced by inactivated VEE complexed with specific homologous IgG at equivalence as previously reported by Houston et al.⁵ in rhesus monkeys. As suggested by Osato⁶ while working with a bacterial complex product, dose-dependency and route of immunization were also investigated as factors of Ag-Ab complex enhancement of the immune response in these multifaceted experiments.

Presently, studies are approaching completion on investigations of dose-dependency and the route of inoculation as factors of enhancement of the immune response in mice to IVEE complexed with specific homologous IgG at equivalence. The IV, IP, SC, and IM routes have been completed. In each study, 3 separate groups of mice were immunized on a one-time basis; 1 group with IVEE vaccine complexed with specific IgG at equivalence and the other 2 groups with appropriate IVEE antigen and IgG antibody controls. The 4 subgroups dosage levels utilized with the IVEE-IgG complex were 0.01, 0.1, 0.3, and 0.5 ml, while the Ag and Ab controls used were appropriately half the dosage levels of the complex. Mice from each group were bled daily for 28 days, and PRN titers were obtained. Table X shows the titers of mice using the IV and IP routes of inoculation. Likewise, Table XI shows results from the SC and IM routes of inoculation. The results suggest that the IVEE-IgG complex formed at equivalence and given IV to mice stimulates an enhanced immune antibody response which may be dose-related. Comparative statistical analysis of the data is now being completed.

Protection studies utilizing the aforementioned mice used in each dose-dependency and route of inoculation project were conducted by challenging the mice with Trinidad strain VEE virus (10^3 MICLD₅₀) on day 31 postinoculation and observed for mortality for 14 days. Percentage protection results illustrated in Tables X and XI, which represent data from these routes, show that 100% protection was achieved at a dosage level of 0.1 ml following immunization with equivalent complexes with the IV route, whereas a dosage level of 0.25 ml is required for 87% protection if the IVEE alone is given via the IV route. The PRN titers of the SC-route study are much lower than the PRN titers of the IV and IP routes; however, the percentage protection of the SC route appears to resemble the protection results from the IP route.

TABLE X. NEUTRALIZING ANTIBODY RESPONSES IN MICE IMMUNIZED WITH IgG, IVEE, AND IVEE-IgG COMPLEXED AT EQUIVALENCE IV AND IP.

VACCINATION CONDITIONS	RECIPROCAL PRN TITERS BY DAYS							% PROTECTION	
	5	11	14	18	21	25	28		
Intravenous									
IgG									
0.005 ml	4	2	4	4	4	4	4	7	
0.05 ml	4	2	2	2	2	2	4	0	
0.15 ml	4	4	4	2	2	2	2	0	
0.25 ml	4	2	4	2	2	2	2	10	
IVEE									
0.005 ml	8	4	2	4	2	2	4	13	
0.05 ml	8	4	4	16	64	64	64	67	
0.15 ml	32	32	16	16	16	8	16	71	
0.25 ml	64	32	32	256	32	256	64	87	
IVEE-IgG									
0.01 ml	16	16	2	64	64	16	64	55	
0.1 ml	128	128	256	256	1024	256	256	100	
0.3 ml	256	1024	1024	1024	256	1024	256	100	
0.5 ml	256	128	256	256	256	1024	256	100	
Intraperitoneal									
IgG									
0.005 ml	4	4	4	4	4	2	2	0	
0.05 ml	4	4	4	4	4	2	2	0	
0.15 ml	4	4	4	4	2	2	4	5	
0.25 ml	4	4	4	4	4	4	4	3	
IVEE									
0.005 ml	4	4	16	4	4	4	4	6	
0.05 ml	16	4	4	2	2	64	64	49	
0.15 ml	64	16	16	64	4	8	64	83	
0.25 ml	16	64	16	64	16	256	64	78	
IVEE-IgG									
0.01 ml	4	2	4	4	2	2	4	8	
0.1 ml	4	16	4	4	4	16	256	54	
0.3 ml	4	16	16	256	64	64	256	79	
0.5 ml	16	8	64	64	64	64	64	91	

TABLE XI. NEUTRALIZING ANTIBODY RESPONSES IN MICE IMMUNIZED WITH IgG, IVEE, AND IVEE-IgG COMPLEXED AT EQUIVALENCE SC AND IM ROUTES OF INOCULATIONS. THE IM ROUTE TITERS ARE RESULT OF 5 SAMPLES PER POINT GEOMETRICAL MEAN FOR EACH POINT.

VACCINATION CONDITIONS (GROUPS AND DOSAGES)	RECIPROCAL DAYS POSTINOCULATION				% PROTECTION
	5	21	25	28	
Subcutaneous					
<u>IgG</u>					
0.005 ml	<4	<4	<4	<4	3
0.05 ml	<4	<4	<4	<4	7
0.15 ml	<4	<4	<4	<4	13
0.25 ml	<4	<4	<4	<4	3
<u>IVEE</u>					
0.005 ml	<4	<4	<4	<4	13
0.05 ml	<4	<4	<4	<4	40
0.15 ml	4	16	8	16	67
0.25 ml	4	4	16	4	83
<u>IVEE-IgG</u>					
0.01 ml	<4	<4	<4	<4	10
0.1 ml	<4	<4	<4	<4	47
0.3 ml	<4	4	8	16	80
0.5 ml	<4	4	16	64	80
Intramuscular					
<u>IgG</u>					
0.005 ml	<4	<4	<4	<4	1
0.05 ml	<4	<4	<4	<4	0
0.15 ml	<4	6	5	6	0
0.25 ml	4	4	4	4	3
<u>IVEE</u>					
0.005 ml	<4	5	<4	4	1
0.05 ml	<4	<4	7	9	45
0.15 ml	<4	7	10	5	53
0.25 ml	4	9	14	10	80
<u>IVEE-IgG</u>					
0.01 ml	<4	10	8	7	52
0.1 ml	6	12	14	18	61
0.3 ml	5	12	37	14	71
0.5 ml	5	12	14	10	68

Collaborative in vivo investigations with LTC Hilmas and LTC Houston concerning vascular clearance studies by the RE system of VEE (TC-83 strain) virus in rhesus monkeys have been concluded. Since ^{125}I , as an extrinsic label for a virus marker, was suspected of possible dissociation of the isotope and virus, an intrinsic label (^3H -AA) was prepared for use in the next studies. However, due to quenching and blood volume dilution in rhesus monkeys, use of ^3H -AA as a viral, radioactive label in rhesus monkeys was not as good as expected. To this end, a ^{32}P radioactive label was prepared and utilized in the clearance studies to obtain higher CPM and to avoid the quenching problem. Initial attempts to count whole blood samples were unsuccessful; thereafter, plasma samples were utilized for determining radioactivity of the cleared samples. Results of these studies are presented in Work Unit 834 01 010.

Current procedures of pertinent methodology of other investigators at the Institute were assessed in our investigations of cellular mechanisms of the immune response. Utilizing a purified cell culture of lymphocytes separated from human peripheral blood, an in vitro lymphocyte viability study was conducted in the Amicon[®] Vitafiber artificial capillary system. A 2.6-ml purified culture containing $4.2 \times 10^6/\text{ml}$ of human peripheral lymphocytes was suspended in RPMI 1640 medium, and counted at 24 and 72 hr. At 24 hr, a 0.2-ml aliquot stained with erythrosin B for viability showed 2×10^3 viable lymphocytes/ml. At 72 hr, 100% mortality was observed in an aliquot stained for viability.

Publications:

1. Harrington, D. G., M. R. Elwell, and D. E. Hilmas. 1976. Observations on the response of monkeys to intranasal challenge with Japanese encephalitis virus. Arthropod-Borne Virus Information Exchange 30:141-142.
2. Harrington, D. G., M. E. Elwell, and D. E. Hilmas. 1976. Response of monkeys to intranasal challenge with Japanese encephalitis virus. Abstracts of the Meeting - 1976. Am. Soc. Microbiol. p. 259.
3. Heard, C. D., D. E. Hilmas, and P. B. Jahrling. 1976. Vascular clearance of Venezuelan equine encephalemyelitis viruses as a determinant of virulence for rhesus monkeys. Abstracts of the Meeting - 1976. Am. Soc. Microbiol. p. 260.
4. Houston, W. E., C. L. Crabbs, R. J. Kremer, and J. W. Springer. 1976. Adjuvant effects of diethylaminoethyl-dextran. Infect. Immun. 13: 1559-1562.
5. Houston, W. E., C. L. Crabbs, E. L. Stephen, and H. B. Levy. 1976. Modified polyriboinosinic-polyribocytidylic acid, an immunological adjuvant. Infect. Immun. 14:318-319.

LITERATURE CITED

1. Field, A. K., A. A. Tytell, G. P. Lampson, and M. Hilleman. 1967. Inducers of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. Proc. Nat. Acad. Sci. USA 58:1004-1010.
2. Devita, V., G. Canellos, P. Garbone, S. Baron, H. Levy, and H. Gralnick. 1970. Clinical trials with interferon (InF) inducer polyinosinic-cytidylic acid (PIC). Proc. Am. Ass. Cancer Res. 11:21.
3. Woodhour, A. F., A. Friedman, A. A. Tytell, and M. R. Hilleman. 1969. Hyperpotentiation by synthetic double-stranded RNA of antibody responses to influenza virus vaccine in adjuvant 65. Proc. Soc. Exp. Biol. Med. 131:809-817.
4. Levy, H. B., G. Baer, S. Baron, C. E. Buckler, C. J. Gibbs, M. J. Iadarola, W. T. London, and J. Rice. 1975. A modified polyriboinosinic-polyribocytidyl acid complex that induces interferon in primates. J. Infect. Dis. 132:434-439.
5. Houston, W. E., C. E. Pedersen, Jr., F. E. Cole, Jr., and R. O. Spertzel. 1974. Effects of antigen-antibody complexes on the primary immune response in rhesus monkeys. Infect. Immunity 10:437-442.
6. Osato, K. 1972. Antigen-antibody complexes in the immune response. I. Analysis of the effectiveness of complexes on the primary antibody response. Immunology 23:545-557.
7. Gerloff, R. K., B. H. Hoyer, and L. C. McLaren. 1962. Precipitation of radiolabeled poliovirus with specific antibody and antiglobulin. J. Immunol. 89:559-570.
8. Hsu, T. C., L. P. Chow, H. Y. Wei, C. L. Chen, S. T. Hsu, C. T. Huang, M. Kitaoka, and H. Sunaga. 1971. Controlled field tests of vaccine in man. A. A completed field trial for an evaluation of the effectiveness of mouse-brain Japanese encephalitis vaccine, p. 258-265. In W. McD. Hammon, M. Kitaoka, and W. G. Downs (eds.), Immunization for Japanese encephalitis. Williams and Wilkins, Baltimore.
9. Lee, G. C.-Y., J. T. Grayston, and S.-P. Wang. 1967. Protective studies in mice and monkeys with an inactivated Japanese encephalitis virus vaccine grown in hamster diploid cell culture. Proc. Soc. Exp. Biol. Med. 125:803-808.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ² DA OE6426	2. DATE OF SUMMARY ³ 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SURVEY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCY ⁴ U	6. WORK SECURITY ⁵ U	7. REGARDING ⁶ NA	8. DOD/INSTRN ⁷ NL	9. SPECIFIC DATA-CONTRACTOR ACCESS ⁸ <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT
10. NO./CODES ⁹	PROGRAM ELEMENT 62760A	PROJECT NUMBER 3A762760A834		TASK AREA NUMBER 02	WORK UNIT NUMBER 423		
B. PRIMARY							
C. CONTRIBUTING							
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11. TITLE (Provide with Security Classification Code) (U) In vitro studies of human immune response to vaccines of military importance							
12. SCIENTIFIC AND TECHNOLOGICAL AREA ¹⁰ 003500 Clinical medicine; 004900 Defense; 010100 Microbiology							
13. START DATE 74 01	14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house			
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C. NUMBER: NA		C. AMOUNT:		CUM. AMT.		135.0	
21. RESPONSIBLE DOD ORGANIZATION							
NAME ¹¹ : USA Medical Research Institute of Infectious Diseases ADDRESS ¹² : Fort Detrick, MD 21701				NAME ¹³ : Virology Division ADDRESS ¹⁴ : USAMRIID Fort Detrick, MD 21701			
PRINCIPAL INVESTIGATOR (Provide DODAN II U.S. Academic Institution) NAME ¹⁵ : Edelman, R. TELEPHONE: 301 663-7241 SOCIAL SECURITY ACCOUNT NUMBER:							
ASSOCIATE INVESTIGATORS NAME: Levitt, N. H. NAME: POC:DA							
22. KEYWORD (Provide EACH with Security Classification Code) (U) BW defense; (U) Military medicine; (U) Virus vaccine; (U) Mitogens; (U) Lymphocyte transformation; (U) Delayed cutaneous hypersensitivity							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Provide rest of each with Security Classification Code.) 23 (U) Establish and standardize in vitro techniques for studying cell-mediated and humoral components of the human immune system in viral infections resulting from attenuated vaccines employed by the military, especially those for BW Defense. 24 (U) Examine in a very detailed way blood specimens collected from at-risk personnel receiving routine immunizations at the Institute. Included will be hematology, antibodies, electrophoresis, B and T cell kinetics and interferon levels. 25 (U) 75 07 - 76 06 - The "active" rosette test was adapted to an in vitro assay for predicting human delayed cutaneous hypersensitivity (DCH) to microbial antigens. Tuberculin or tularemia skin test antigen, incubated with lymphocytes from antigen-sensitive donors, produced a significant increase in the ability of the lymphocytes to form active rosettes when compared to the percent active rosette forming cells (A-RFC) in companion lymphocytes cultured without antigen. The assay appears to be specific for the antigen to which the individual demonstrates DCH. This assay may provide a new in vitro method for investigating mechanisms of cell immunity and a rapid diagnostic test for sensitization to microbial antigens. Investigations of the interaction of human peripheral blood leukocytes and TC-83-VEE virus have shown that macrophages support maximum virus growth, that only low grade virus replication occurs in lymphocytes, and that polymorphonuclear leukocytes do not support virus replication. Addition of phytohemagglutinin to lymphocyte cultures enhances virus yields 10,000 times. Publications: Program, 15th Intersci. Conf. Antimicrob. Agents Chemother., Nos. 133 and 134, 1975. J. Immunol. 116:1110-1114, 1976. In: Malnutrition and the Immune Response, 1976, 2 papers, in press.							
Available to contractors upon contractor's approval.							

DD FORM 1498 1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

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BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 423: In vitro Studies of Human Immune Response and to Vaccines of Military Importance

Background:

We have adapted and developed lymphocyte assays to examine the interaction of cellular and humoral immune responses after vaccination or natural infection.

The human cell-mediated immune system is populated by thymus-derived lymphocytes or T cells. The ability of a lymphocyte to spontaneously bind sheep erythrocytes (SE) in a rosette formation is one way to identify and measure T cells. Such a subpopulation has been identified which possesses high affinity receptors for SE. These cells are referred to as active rosette forming cells (A-RFC); it is thought that A-RFC may be the effector T cells.¹ We have recently reported a significant rise in A-RFC in skin test positive individuals within 48 hr of skin testing with microbial antigens.² Skin test nonresponders exhibited no significant rise in A-RFC.² We hypothesized that the skin test antigens in some manner alter T-lymphocyte membranes in sensitized persons to permit more avid binding of SE. This report summarizes our successful attempts to develop a test that would detect the same change in lymphocyte cultures. Preliminary data on this test were summarized last year.

A preliminary study was initiated to investigate the interaction of group A arboviruses and human peripheral blood leukocytes (PBL), which have been shown to support growth of TC-83 virus in vitro.

Progress:

In vitro A-RFC Studies. Purified lymphocytes from 3 tuberculin-positive donors were incubated with and without PPD at 37 C for various time intervals in order to determine whether any change in the percentage of A-RFC occurred. A PPD dose of 100 ng/ml was chosen because this concentration approximates that employed in the in vitro lymphocyte transformation assay. The cells were assayed for their ability to form active rosettes after 1, 2, 4, and 6 hr incubation. The lymphocytes incubated with PPD exhibited a significant increase (> 2 SD) in their ability to form active rosettes; the optimum incubation time appeared to be 4 hr. No rise in % A-RFC occurred in the absence of PPD antigen. A similar rise in % A-RFC occurred after 4 hr in lymphocyte cultures of one tularemia, skin-test positive individual, using a tularemia antigen dose of 160 ng/ml.

Dose-response experiments were performed to determine the optimum concentration of antigens to use for the assay. Lymphocytes from 7 PPD skin-test positive donors were incubated with log₁₀ serial dilutions of PPD antigen ranging from 0 to 0.01-100 ng/ml. The antigen dose giving the maximum increase in A-RFC after 4 hr was 100 ng/ml. Significant but lesser increases were also observed with the 10 ng/ml dose.

A dose-response curve was determined for 2 tularemia positive donors, using \log_{10} serial dilutions of tularemia antigen ranging from 0, to 0.16-1600 ng/ml. Three antigen concentrations (1.6, 16, 160 ng/ml) appeared to stimulate a significant rise in % A-RFC, with 160 ng/ml appearing to be optimum. The antigen concentration of 1600 ng/ml was cytotoxic to the lymphocytes.

In order to ascertain the specificity of the in vitro A-RFC test, lymphocytes drawn from 3 tuberculin and 2 tularemia-negative individuals were incubated with concentrations of specific antigens used to culture positive individuals. None of the antigen doses stimulated a rise in A-RFC after 4 hr in these donors.

TABLE I. DELAYED CUTANEOUS HYPERSENSITIVITY AND IN VITRO A-RFC ASSAY RESULTS IN PERSONS TESTED WITH ONE ANTIGEN

DONOR NO.	SKIN TEST INDURATION (mm)		% Δ IN A-RFC	
	PPD	Tularemia	Initial	Repeat
Donor lymphocytes incubated with PPD, (100 ng/ml)				
1	0 x 0	-	+13	0
2	0 x 0	-	-15	
3	0 x 0	-	- 6	-22
4	10 x 12	-	+28	+67
5	20 x 20	-	+70	+55
6	16 x 16	-	+33	+16
7	20 x 20	-	+24	
8	19 x 24	-	+64	
Donor lymphocytes no. 9-13 incubated with tularemia antigen (160 ng/ml)				
9	-	0 x 0	-53	
10	-	0 x 0	-36	
11	-	0 x 0	-23	
12	-	5 x 6	+80	
13	-	12 x 15	+95	

We next attempted to correlate the results of the in vitro A-RFC assay and the skin test responses of the same lymphocyte donors; 20 donors were skin tested with PPD or tuberculin. Their delayed cutaneous hypersensitivity (DCH) responses measured in mm induration at 48 or 72 hr, are shown in Tables I and II. The % A-RFC in paired antigen-stimulated and control cultures was measured using the optimum time and antigen concentrations. The results are expressed in Tables I and II as the percent rise or fall of A-RFC in the antigen-stimulated culture compared to A-RFC in the companion control

culture. Each culture was run in triplicate and the results averaged. In many cases, repeat A-RFC tests were run 4 - 12 months later. Seven donors had skin tests with both tuberculin and tularemia antigens (Table II). In 9 skin-test negative donors, the change in the proportion of active rosetting lymphocytes in the presence of homologous antigen ranged from -53 to +13%. By contrast, in 13 of the 14 skin-test positive donors the change in % A-RFC induced by homologous antigen ranged from +16 to +104%.

TABLE II. DELAYED CUTANEOUS HYPERSENSITIVITY AND IN VITRO A-RFC ASSAY RESULTS IN PERSONS TESTED WITH TWO ANTIGENS

DONOR NO.	PPD			Tularemia		
	Skin Test Induration (mm)	% Δ in A-RFC		Skin Test Induration (mm)	% Δ	
		I	R		I	R
14	0 x 0	-6	-3	10 x 12	+56	+95
15	0 x 0	-11		8 x 9	+104	
16	0 x 0	-6	+6	15 x 15	+70	
17	11 x 14	+23	+71	10 x 12	+44	
18	14 x 15	+32	+103	8 x 8	+70	+85
19	5 x 7	+71	+93	10 x 12	+53	
20	20 x 30	-37	-24	9 x 9	+0	

I = initial; R= repeat See Table I

We assumed from these data a change in A-RFC > +15% following incubation with PPD or tularemia to be indicative of lymphocyte sensitivity to the antigen. The lymphocytes from 1 donor (#20, Table II), who was skin-test positive to tuberculin and tularemia did not show a rise to either antigen in the A-RFC test. These results indicate that the in vitro A-RFC was reasonably immuno-specific. It was reproducible in the 11 donors tested more than once with the same antigen. In 19 of 20 donors, the A-RFC confirmed the skin test sensitivity to 1 or both bacterial antigens. This assay provides a new in vitro method for investigating mechanisms of cell-mediated immunity (CMI) and a rapid diagnostic test for sensitization to microbial antigens.

The biological nature of the observed reaction is unknown. One possibility involves the release of a soluble factor or factors from the antigen-specific lymphocytes after contact with antigen, which in turn is capable of altering the membrane receptors of other T lymphocytes so they also have a higher affinity for sheep RBC. This possibility was tested in several pilot studies. Supernatants were collected from cultures of sensitized lymphocytes incubated with specific antigen for 4 hr. When nonresponsive lymphocytes from skin-test negative donors were incubated with these supernatants the % of A-RFC increased significantly.

Suitable control supernatants did not produce a rise in % A-RFC. Thus it would appear that a soluble factor(s) may be released upon incubation of sensitized lymphocytes with antigen which can convert a nonrosetting cell into an active rosetting cell.

In pilot studies, we examined whether the lymphocyte mitogen, phytohemagglutinin (PHA), could also increase the % A-RFC in vitro. Within several minutes after adding 1.0 - 100 mg PHA to lymphocyte cultures the % A-RFC rose from 21 - 29% to 57 - 71% in a direct dose-response fashion. The induction of new A-RFC could be reversed to control levels by washing the cells exhaustively after PHA was added. PHA did not induce or enhance rosetting of erythrocytes, that do not normally bind well to human lymphocytes, such as cells from the goose, chicken, rabbit, human, or goat; the enhanced binding seems specific for sheep RBC. The mechanism for the very rapidly enhanced binding of sheep erythrocytes to human lymphocytes is under study. Thus far it does not appear to be a consequence of increased nonspecific "stickiness" of cells exposed to PHA. Further studies, including use of other mitogens, are planned.

Unfortunately, the in vitro A-RFC test, as now performed, can be satisfactorily reproduced by only 1 person in this laboratory. In order to standardize the test for more routine laboratory use, we explored many ways to improve its precision and reproducibility. During the course of our studies we developed a "mechanized" A-RFC test, that uses a rapidly reciprocating shaker instead of a Pasteur pipette to resuspend the pelleted, lymphocyte-sheep erythrocyte rosettes. The resuspension of rosettes represents the critical step in the procedure. The shaker repeatedly provided good agreement between replicates of the same sample. The ability of the mechanized procedure to detect biological changes in % A-RFC was next evaluated. Lymphocytes from 22 persons were tested in vitro. The number of tests done per person ranged from 1 to 17; a total of 150 tests were performed using PPD and tularemia antigens incubated with lymphocyte cultures for 2 - 72 hr before the % A-RFC was measured. Initially any rise above control values was taken as a marker of lymphocyte sensitivity to antigen. A rise in % A-RFC was only found in 49 of 84 tests of skin-test positive donors (58%) and in 19 of 47 tests of skin-test negative donors (40%; $\chi^2 = 3.41$, $0.5 < P < 0.1$). The data were recalculated using an increase of $\geq 10\%$ above control A-RFC values as the criteria for antigen sensitivity; 45 and 17% of tests were then positive in sensitized and nonsensitized donors, respectively ($P = 0.047$). Although significantly more positive tests were found in sensitized persons, it was clear that many of these positive donors would be considered not sensitized on the basis of their in vitro test results. Thus, although the results suggest the mechanized variation of the A-RFC test measures a biological phenomenon, it is not sufficiently sensitive to permit its use as a routine in vitro test for cellular immunity.

In vivo A-RFC Studies. We attempted to determine at what point in the first 24 hr after skin testing the % A-RFC rises. Two volunteers previously vaccinated with tularemia vaccine were bled every 6 hr from 0800 - 2000 hours for 7 bleedings. Tularemia skin tests were done after the 4th bleeding. The % A-RFC rose significantly above the 4 base-line values between 12 and 24 hr

after skin testing. The results also showed that in both volunteers there was no variation in the % A-RFC over time, but there was an evening rise in the absolute number of peripheral lymphocytes.

Studies of Virus-Leukocyte Interaction. Macrophages have been shown to be an important factor in generalized viral infections. The fixed and wandering histiocytes remove virus particles from the blood, the serous cavities, the respiratory tract and from the connective tissues. Depending on the nature of the virus, and the species of host animal, these macrophages may either destroy the virus or support its multiplication. In many viral diseases such as poxvirus infections circulating monocytes act as the vehicle of viremia. In nonimmune mice, the Kupffer cells rapidly remove ectromelia virus from the circulation; subsequently the virus multiplies in these cells and spreads from them to the parenchymal cells of the liver. Recent studies have demonstrated that viruses differ in their ability to replicate in diverse types of human leukocytes. Certain viruses, like measles, VSV, polio and 17 D strain of yellow fever have been shown to replicate in human macrophages, while herpes, EBV and CMV only replicate in human lymphocytes. When lymphocytes are stimulated with the non-specific mitogen, PHA, several viruses demonstrated enhanced growth.

Little information is available on the interaction of group A arboviruses with human PBL. A preliminary study was undertaken to first determine whether PBL were capable of supporting TC-83 virus growth and subsequently which subpopulation of leukocyte was involved. PBL were separated from heparinized whole human blood by incubation at 37 C with 5% dextran. The washed PBL were infected with TC-83 virus (MOI = 1), washed with BSS and resuspended in RPMI - 1640 medium at a concentration of 10^6 cells/ml. The PBL culture was incubated at 37 C. Samples for virus assay were taken daily and titrated on DEC monolayers. This PBL culture yielded titers of 6×10^3 , 4×10^5 , 1×10^6 , 1×10^6 , 6×10^5 on days 0 to 4, respectively. It was evident that these cells could support TC-83 virus growth.

The next experiments were performed to determine which leukocyte subpopulation(s) was responsible for the significant virus growth. Mononuclear cells (monocyte, lymphocyte) were separated from PMN cells by centrifugation of PBL on a hypaqueficol gradient. Both cell types were harvested, washed and infected with TC-83 virus as described above. It is obvious from the results that the mononuclear fraction was responsible for virus growth (Table III).

In the next experiment, the mononuclear fraction was further differentiated into separate macrophage and lymphocyte cultures by absorbing the macrophages to a plastic surface. Residual macrophages present in the lymphocyte culture were killed by the addition of silica, a substance shown by several investigators to selectively destroy macrophages.³ PHA was added to a lymphocyte culture to determine its effect on virus growth. These cultures were infected and a virus growth study performed.

Results shown in Table IV indicate that more growth was observed in the macrophage culture, than in the macrophage-free lymphocyte culture which demonstrated only minimal virus replication. However, the greatest yield of virus occurred in PHA stimulated lymphocyte cultures.

TABLE III. IN VITRO TC-83 VIRUS GROWTH IN FRACTIONS OF HUMAN PERIPHERAL BLOOD LEUKOCYTES (PBL)

PBL FRACTION	PFU/ML BY DAY POSTINFECTION				
	0	1	2	3	4
Mononuclear	5×10^1	6×10^5	9×10^6	1×10^7	3×10^5
PMN	4×10^3	2.5×10^1	0	0	0

TABLE IV. IN VITRO REPLICATION OF TC-83 VIRUS IN HUMAN LEUKOCYTE CELL TYPES

CELL TYPE	PFU/ML BY DAY POSTINFECTION				
	0	1	2	3	4
Macrophage	1.5×10^2	1×10^6	1×10^6	1×10^4	1×10^3
Macrophage + silica	1×10^2	1×10^2	0	0	0
Lymphs	6×10^2	1×10^5	2.5×10^4	5×10^3	1×10^3
Lymphs + silica	3×10^3	1×10^4	5×10^2	2.5×10^2	3×10^2
Lymphs + silica + PHA	1×10^4	1×10^5	5×10^6	1.5×10^6	2×10^6
None, virus control	3×10^4	1×10^3	1×10^3	2×10^2	1.5×10^2

Additional studies of the interaction of group A arboviruses with human PBL are presently in progress.

Presentations:

1. Felsburg, P. J., and R. Edelman. The active E-rosette test: a sensitive in vitro assay for human delayed type hypersensitivity to microbial antigens. Presented at, 15th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, 24 Sep 1975 (Program and Abstracts, no. 133).

2. Edelman, R. The active E-rosette test. Presented at, Seminar at Walter Reed Army Institute of Research, Washington, DC, 7 Oct 75.

Publications:

1. Edelman, R., and P. J. Felsburg. 1975. The active E-rosette test: correlation with delayed cutaneous hypersensitivity. Program and Abstracts, 15th Interscience conference on Antimicrobial Agents and Chemotherapy, Washington, DC, 24-26 Sep 1975, no. 134.
2. Felsburg, P. J., R. Edelman, and R. H. Gilman. 1976. The active E rosette test: correlation with delayed cutaneous hypersensitivity. *J. Immunol.* 116:1110-1114.
3. Edelman, R. 1976. Cell-mediated immune response in protein-calorie malnutrition--A review. *In* *Malnutrition and the Immune Response* (ed. R. M. Suskind), Raven Press, New York, in press.

LITERATURE CITED

1. Wybran, J., and H. H. Fudenberg. 1973. Thymus-derived rosette-forming cells in various human disease states: cancer, lymphoma, bacterial and viral infections, and other diseases. *J. Clin. Invest.* 52:1026-1032.
2. Felsburg, P. J., R. Edelman, and R. H. Gilman. 1976. The active E-rosette test: correlation with delayed cutaneous hypersensitivity. *J. Immunol.* 116:1110-1114.
3. Allison, A. C., J. S. Harrington, and M. Birbeck. 1966. An examination of the cytotoxic effects of silica on macrophages. *J. Exp. Med.* 124:141-154.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION DA OF6417	2. DATE OF SUMMARY 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636		
3. DATE PREV SUBJPT 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SECY ^b U	6. WORK SECURITY ^b U	7. REGRADING ^c NA	8. DISSEMIN INSTRN ^c NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM- A WORK UNIT	
10. NO./CODES: ^a B. PRIMARY 62760A	PROGRAM ELEMENT PROJECT NUMBER 3A762760A834			TASK AREA NUMBER 02	WORK UNIT NUMBER 425			
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11. TITLE (Provide with Security Classification Code) ^b (U) Biophysical and biochemical characterization of arenaviruses and their structural components								
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19. RESPONSIBLE DOG ORGANIZATION NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701	20. PERFORMING ORGANIZATION NAME: Virology Division USAMRIID ADDRESS: Fort Detrick, MD 21701							
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833	PRINCIPAL INVESTIGATOR (Provide SEAN // U.S. Academic Institution) NAME: Gangemi, J. D. TELEPHONE: 301 663-7241 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME:							
21. GENERAL USE Foreign intelligence considered			POC:DA					
22. REVISIONS (Provide with Security Classification Code) (U) Arenaviruses; (U) Biophysics; (U) Biochemistry; (U) Electron microscopy; (U) RNA viruses; (U) BW defense; (U) Military medicine; (U) Vaccine development								
23. TECHNICAL OBJECTIVE ^a 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Provide rest of each with security Classification Code.) 23 (U) Isolate and characterize by physical and biochemical techniques virion sub-populations of arenaviruses in order that their infection and structural characteristics may be better understood; identify polypeptides of arenavirus virion which have potential as subunit vaccines for diseases of military importance in BW Defense.								
24 (U) Prepare large quantities of selected viruses; concentrate and study them by a variety of biophysical and biochemical means. Infectivity of subpopulations will be assayed by appropriate techniques.								
25 (U) 75 07 - 76 06 - The polypeptide composition of Pichinde, Tacaribe and Machupo viruses has been determined. Biochemical analysis of these selected arenaviruses revealed 3 common MW classes of polypeptides (65,000, 35,000 and 13,000 daltons). One additional polypeptide species at 12,000 appeared in both Machupo and Tacaribe virus preparations, while a second with a MW of 45,000 was found only in Tacaribe virions. Defective Pichinde particles were isolated in substantial quantities with the aid of a large volume zonal rotor. These defective particles were (a) half the size of standard virions, (b) interfered with the plaque production of standard virions, and (c) were 500 times less infectious than standard virions as determined by particle:PFU ratios. Virus-incorporated ribosomes have been isolated from highly concentrated arenavirus stocks and examined with several biophysical techniques. Results from these studies revealed that many of these ribosomes were specifically associated with the virus envelope. Those ribosomes which were not envelope associated, existed as unbound polyribosomes inside the virus particles. Contour length measurements of unbound polyribosomes revealed a heterogeneous mixture of sizes. No consistent correlation between polyribosome size and genome segment size was made.								
Publication: Am. Soc. Microbiol. Abstracts of the Meeting -- 1976, p. 218.								

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 425: Biophysical and Biochemical Characterization of Arenaviruses and Their Structural Components

Background:

Thin-sections of infected cells and negative stains of concentrated virions have revealed morphological differences between particles of arenavirus populations. Most apparent in these differences is the variation in particle size.¹ The significance of these size differences with respect to infectivity and virulence is unknown. Nonetheless, in light of the fact that both multiple pieces of virus-specific RNA and host specific ribosomes are incorporated into virions during maturation, it appears likely that the nonuniform incorporation of one or both of these elements could result in particle heterogeneity. Such a hypothesis can be tested if the virion subpopulations which differ in size can be fractionated and then concentrated in order that standard biochemical analysis can be performed.

The biochemical relationships between pathogenic and nonpathogenic arenaviruses are not known. It has however been reported that some nonpathogenic members induce immunity to infection with pathogenic varieties.² Since we are able to concentrate and purify both pathogenic and nonpathogenic varieties in our laboratory facilities, the biochemical characterization of virus members from both groups is a realistic goal. Such studies could result in the identification of the structural polypeptides responsible for the induction of neutralizing antibodies.

Progress:

Virion subpopulations which differ from one another in particle size and sedimentation rates were fractionated in a large volume zonal rotor. The slow sedimenting 90-nm particles were found to be defective (only 1 particle in 500 was capable of producing a plaque) and also interfered with the production of standard virions. The larger, fast sedimenting, 200 nm particles were 500 times more infectious (particle:PFU ratio) than the smaller defective particles and appeared to be more regular in shape when closely examined in the electron microscope. We are now examining the biochemical properties of both the small and large particle subpopulations.

Comparison of the polyacrylamide gel profiles of the structural polypeptides from 2 nonpathogenic arenaviruses, Pichinde and Tacaribe, with those of a pathogenic member, Machupo, revealed a common set of structural proteins. These polypeptides fell into three MW size groupings, 65,000, 35,000 and 13,000. Each of the 3

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arenaviruses shared these polypeptide similarities. However, Machupo had an additional polypeptide species at 12,000 while Tacaribe had a 45,000 as well as the 12,000 polypeptide species. The immunologic activity of specific polypeptide species is now being examined in an attempt to identify the antigens responsible for the induction of neutralizing antibody.

Virion-incorporated ribosomes have been isolated from highly concentrated arenavirus stocks and examined with several biophysical techniques. Results from these studies revealed that virion-incorporated ribosomes were identical to host-cell ribosomes isolated from uninfected cells. Some of the virion-incorporated ribosomes were specifically associated with the viral envelope, while those which were not, existed as unbound polyribosomes inside the virus particles. Contour length measurements of unbound polyribosomes revealed a heterogeneous mixture of sizes. No consistent correlation between polyribosome size and genome segment size was made.

Presentation:

Gangemi, J. D., E. M. Johnson, R. R. Rosato, and G. A. Eddy. Biochemical analysis of the polypeptide composition of several arenaviruses. Presented, American Society for Microbiology, Atlantic City, NJ. 2-7 May 1976. (Abstracts of the Meeting - 1976, p. 218).

LITERATURE CITED

1. Murphy, F. A., P. A. Webb, K. M. Johnson, S. G. Whitfield and W. A. Chappell. 1970. Arenoviruses in Vero cells: ultrastructural studies. J. Virol. 6:507-518.
2. Parodi, A. S., and C. E. Coto. 1964. Immunizacion de cobayos contra el virus Junin por inoculacion del virus Tacaribe. Medicina (B. Air.) 24:151-153.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ¹	2. DATE OF SUMMARY ²	REPORT CONTROL SYMBOL	
3. DATE PREV SUBM ³ 76 02 12	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCITY ⁴ U	6. WORK SECURITY ⁵ U	7. REGRADING ⁶ NA	8. ORIGIN INSTRN ⁷ NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT
10. NO./CODES: ⁸ PROGRAM ELEMENT PROJECT NUMBER				TASK AREA NUMBER		WORK UNIT NUMBER	
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b. CONTRIBUTING							
c. <i>[scribbled]</i>	CARDS 114(e) (f)						
11. TITLE (Provide with Security Classification Code) ⁹ (U) Arenavirus-rodent models for use in studies of cross-protection							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ¹⁰ 003500 Clinical medicine; 004900 Defense; 010100 Microbiology							
13. START DATE 76 01	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house				
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a. DATES/EFFECTIVE:				b. PROFESSIONAL MAN YRS			
b. NUMBER: ¹¹ NA				FISCAL YEAR	76	0.3	21
c. TYPE:				c. CURRENT			
d. KIND OF AWARD:				77	1.0	121	
e. CUM. AMT.				f. FUNDS (in thousands)			
19. RESPONSIBLE DOG ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ¹² USA Medical Research Institute of Infectious Diseases ADDRESS: ¹³ Fort Detrick, MD 21701				NAME: ¹² Virology Division USAMRIID ADDRESS: ¹³ Fort Detrick, MD 21701			
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833				PRINCIPAL INVESTIGATOR (Provide DOB if U.S. Academic institution) NAME: Rosato, R. R. TELEPHONE: 301 663-7241 SOCIAL SECURITY ACCOUNT NUMBER:			
21. GENERAL USE Foreign intelligence considered				ASSOCIATE INVESTIGATORS NAME: Cole, Jr., F. E. NAME: Jahrling, P. B. POC:DA			
22. KEY WORDS / Code words each preceded by security classification code (U) Lethal rodent models; (U) Cross-protection; (U) Arenaviruses; (U) Tacaribe complex (U) Military medicine; (U) BW defense; (U) Vaccines; (U) Antiviral chemotherapy							
23. TECHNICAL OBJECTIVE, ¹⁴ 24. APPROACH, 25. PROGRESS (Provide text of each with Security Classification Code.) 23 (U) Develop and define rodent models of lethal infection for use in arenavirus studies; investigate cross-protection conferred by Tacaribe complex viruses against lethal challenge in the developed models; obtain basic data pertinent to cross-protection among arenaviruses for possible vaccine development, employ the lethal model as an indirect system for testing the efficacy of antiviral compounds against virus diseases of potential BW importance. 24 (U) Adult rodent models of lethal arenavirus infection will be developed by sequential passage of infected brain material to obtain strains of increased lethality. Models will be examined as to time of onset, degree and duration of protection afforded by members of the Tacaribe complex against challenge with the developed virus strains of increased lethality. 25 (U) 76 01 - 76 06 - Isolates of Tacaribe virus strain 11573 have been obtained that exhibit approximately 85% mortality by the intracerebral (IC) route in adult mice. Sufficient quantities of these isolates are available for use in cross-protection studies and for use in other rodents to develop usable adult models of arenavirus infections. Cross-protection studies have been started; studies to determine onset, duration and degree of protection afforded by viruses of the Tacaribe complex against lethal IC challenge are in progress. Ancillary studies as to presence of viremia, temporal growth of protecting viruses in body fluids and tissues, humoral antibody responses and effect of immunosuppressive agents on protection are in progress. Publication: Toxicol. Appl. Pharmacol. 35:107-111, 1976.							

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AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

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BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A766760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 426: Arenavirus Rodent Models for Use in Studies of Cross-Protection

Background:

Currently recognized arenaviruses are lymphocytic choriomeningitis (LCM), Lassa fever, Tacaribe, Pichinde, Tamiami, Amapari, Latino, Parana, Machupo and Junin viruses; all viruses but LCM and Lassa fever are grouped together in the Tacaribe complex.¹ Many of these viruses produce chronic infections in natural rodent hosts in which long-term viremias with virus shedding in the urine are important factors in transmission from rodent to rodent. Virus shedding may also be important in man to man infection with Machupo and Junin viruses. In man, both Machupo and Junin viruses cause acute, hemorrhagic fever, characterized by leukopenia, gastrointestinal hemorrhage, shock, and transitory neurological signs. Mortality varies from 10 - > 50%. Human infections with Tacaribe, Parana, Tamiami, and Amapari viruses are unknown, although all infect rhesus monkeys. None of the viruses of the Tacaribe complex are pathogenic for adult rodents as originally isolated from nature. Therefore high priority has been given to adapting arenaviruses to adult rodents to facilitate cross-protection studies using survival as the indicator of the degree of cross-protection. Animal models are already available for the most virulent human viruses, Machupo and Junin, both of which require specialized containment facilities. To date there are no lethal rodent models for the remaining viruses of the Tacaribe complex. The requirement for these models stems from the reported observations that immunization with Tacaribe virus affords animals protection against lethal challenge with Junin² and Machupo³ viruses, a clear indication that cross-protection may be a possible means of protecting humans against these lethal arenaviruses.

Progress:

A preliminary study was done to ascertain if cross-protection could be demonstrated among members of the Tacaribe complex of arenaviruses. Six arenaviruses were given SC to mice in an attempt to protect them against later intracerebral (IC) challenge with Tacaribe virus. Mice 21-23 days old were given a single SC dose of 10^3 PFU of arenaviruses Amapari, Parana, Pichinde, Tacaribe, Tamiami, or Latino either 13 or 5 days prior to IC challenge with 10^5 PFU of Tacaribe virus. When inoculation preceded challenge by 13 days, Amapari, Pichinde and Tamiami viruses elicited 30% protection and Latino virus only 10%, ($p < 0.05$). In contrast Parana and Tacaribe viruses conferred 60% ($p < 0.01$) and 100% ($p < 0.005$) protection respectively against challenge with Tacaribe virus. Those mice given the SC dose of viruses 5 days prior to challenge exhibited no protection, indicating that the protection observed

with the day-13 regime is probably not due to interfering virus present at the time of challenge. Unprotected controls responded with 18 of 19 and 18 of 20 deaths in the 13 and 5 day groups, respectively, when challenged with the same dose of Tacaribe virus.

We next examined the duration of cross-protection afforded 21-23 day old mice by SC inoculation of either Parana or Tacaribe viruses. Mice were given a single SC dose of either Parana or Tacaribe virus, held 14 days and then were challenged IC with Tacaribe virus at day 0 (14 days) day 7 (21 days), and day 14 (28 days postprotective SC dose). Normal controls were also challenged at each time interval to examine the effect of age on the lethality of the challenge virus (Table I).

TABLE I. PROTECTION OF MICE GIVEN A SINGLE SC DOSE OF PARANA^a OR TACARIBE^b VIRUS AND A SUBSEQUENT IC TACARIBE VIRUS CHALLENGE^c

DAYS POST SC DOSE	GROUP	DEAD/TOTAL	% SURVIVAL	MTD DAYS	ADJUSTED % SURVIVAL ^d
<u>35-37 Days old</u>					
14	Parana	2/9	78	6.5	64
	Tacaribe	0/6	100		86
	Virus controls	12/14	14	8.5	
<u>42-44 Days old</u>					
21	Parana	2/9	78	9.0	42
	Tacaribe	0/6	100		64
		9/14	36	10.4	
<u>49-50 Days old</u>					
28	Parana	2/10	80	6.5	24
	Tacaribe	0/7	100		44
	(49/50) ^d	7/16	56	9.1	

^aSC dose 3800 PFU

^bSC dose 3500 PFU

^c3.6 x 10⁵ PFU.

^d% survival - % survival of age controls.

As indicated a single SC dose of either Parana or Tacaribe virus affords age-related protection against IC challenge with Tacaribe virus for at least 28 days after the protective dose. The increased resistance of aging mice to IC challenge with Tacaribe virus interferes with interpretation of the data. Before any long-term cross-protection studies can be conducted that are interpretable and meaningful, it is necessary to have Tacaribe virus populations, uniformly lethal to adult mice by the IC route.

Since little data are available from the literature, the influence of age on the resistance of mice to IC challenge with Tacaribe virus (strain 11573) was investigated further. Groups of 100 mice 21, 31, 41, 51, and 61 days old were divided into 5 groups of 20 each and given an IC dose of Tacaribe virus containing 10^5 , 10^4 , 10^3 , 10^2 , or 10^1 PFU. The % dead, and mean time to death (MTD) were calculated for each age and dosage level as well as an LD_{50} for each age group. Generally, resistance increased with age, but no differences were noted in the MTD. The $\log_{10} LD_{50}$ by age group were 2.1, 1.4, 0.9, 1.1, and 1.0 respectively confirming the low lethality of Tacaribe virus for adult mice.

We therefore attempted to select a population of Tacaribe virus (11573) with increased lethality for adult mice when inoculated IC. We serially passaged Tacaribe virus in the brains of 75-to-100-day old mice, harvesting the brains when the mice were moribund, and repassaging those brains. Lethality generally increased with subsequent passage and presently is approximately 75-85% at either the 3rd to 5th passage levels. We attempted to further increase this lethality by employing Specific-Pathogen-Free (SPF) mice. Four 2nd passage isolates, highly lethal for conventional mice, were selected for one additional passage in SPF mice. There were no apparent differences in the % mortality determinations between 3rd passage isolates in either conventional or SPF mice.

Both the initial Tacaribe seed stock and isolates of increased lethality have been inoculated IC into adult mice for clinical and pathological examination. Infected mice first exhibit ruffed fur, a hunched position and increased excitability. CNS involvement progresses by day 6 - 8 postinoculation to include hind-limb extension, paralysis and eventual death by day 12 - 15. Pathological examination indicates meningoencephalitis (multifocal, perivascular, nonsuppurative, with neuronal necrosis being most prominent in hippocampal gyri) and gliosis.

The initial Tacaribe virus seed stock 11573 and all adult mouse 2nd passage isolates have been verified as Tacaribe viruses by both CF and PRNT, and free of PPLO and LCM virus by Virology Division personnel. Selected sera from surviving mice infected with parent, 2nd and 3rd passage isolates were examined by a commercial testing laboratory for the presence of 11 adventitious murine agents. All samples were free of both LCM and GD VII viruses, the only adventitious viruses that elicit clinical signs which could be confused with those of Tacaribe virus.

Two additional strains of Tacaribe virus, 15007 and 12498, were passaged in certified DBS-10 cell cultures. First passage of each strain produced pools that titered 10^5 PFU/ml. These new strain preparations were used as test reagents in additional identification tests which further verified the identities of our parent and passage isolate seed stocks. Parent virus Tacaribe strain 11573, and strains 15007 and 12498 were used to immunize 21-23- and 77-day-old mice prior to IC challenge with 11573 and 2 second-passage isolates of increased lethality for aged mice. Table II summarizes the data. A single protective dose of any of the three Tacaribe virus strains afforded young mice 20 - 100%

TABLE II. PROTECTION OF FEMALE MICE^a GIVEN A SINGLE 0.1-ML SC DOSE OF 3 STRAINS OF TACARIBE VIRUS AGAINST SUBSEQUENT 0.03-ML IC CHALLENGE WITH PARENT AND PASSAGE ISOLATES

		YOUNG MICE (n=15)			OLD MICE (n=15)	
STRAIN		% SURVIVAL	MTD days	% SURVIVAL	MTD days	
SC	IC					
11573 ^b	11573 ^e	93	8.0	87	~ 10.0	
15007 ^c		100	-	93	9.0	
12498 ^d		80	9.7	93	9.0	
Control		20	8.3	69	4.1	
11573	Ap ² ^f	93	13.0	100	-	
15007		73	13.5	69	13.8	
12498		20	12.4	69	13.7	
Control		0	10.7	23	10.8	
11573	4p ² ^g	93	12.0			
15007		80	11.3			
12498		53	9.7			
Control		0	10.7			
11573	Pool B ^h			92	~ 4.0	
15007				86	8.0	
12498				87	12.0	
Control				33	12.1	

^aYoung = 21-23 day old; old = 77-day-old.

^bYoung = 1.1×10^3 PFU; old = 1.5×10^6 PFU.

^cYoung = 2.2×10^4 PFU; old = 2.8×10^4 PFU.

^dYoung = 7.8×10^3 PFU; old = 6.8×10^3 PFU.

^eYoung = 1.0×10^5 PFU; old = 1.5×10^5 PFU.

^fYoung = 8.4×10^2 PFU; old = 1.8×10^3 PFU.

^gYoung = 1.3×10^3 PFU.

^hOld = 5.7×10^3 PFU.

protection over controls depending on the challenge virus used. Strain 15007 consistently afforded more protection than 12498 although it must be noted that the protective dose of 12498 was somewhat less in all cases. Isolates Ap² and 4p² killed 100% of the young control mice whereas parent 11573 produced only 80% mortality. It should be noted that the isolates were used at challenge doses 2-3 logs lower than the parent virus. In the old mice protection varied from ~ 18 - 77% over controls. The previously reported observation that parent virus 11573 has decreased virulence for older mice was further confirmed. The Ap² isolate also showed a decreased virulence with the increasing age of the mice.

Parent strain 11573 and virulent isolates obtained by passage in adult mouse brains have therefore been identified as Tacaribe virus by the protection afforded against them in mice by less virulent Tacaribe virus strains 15007 and 12498.

The variation in the degree of protection afforded by 15007 and 12498 against 11573 and isolates of increased lethality is unexplained at this time but may be a reflection of the variation in both protective and challenge dosages or may, in fact, represent actual antigenic strain variation. The dosages are to be standardized by using either suckling hamster or suckling mouse in future studies. The possibility of strain variation can be hopefully resolved by homologous and heterologous cross-protection studies.

To better understand the development of Tacaribe virus infection caused by strain 11573, suckling mice (SM) and suckling hamsters (SH) were inoculated with 10^{-1} through 10^{-4} PFU/0.03 ml IC dose for the purpose of LD₅₀ and MTD determinations, and for the measurement of the temporal growth of virus in the brain. Paired litters were employed in which MTD and % mortality determinations were obtained from one litter and the other litter sequentially sacrificed to allow virus titers of brain homogenates to be determined.

LD₅₀ determinations could not be calculated because > 50% mortality occurred at the lowest dilution of virus used. Maximum PFU titers in SM (10^5) were generally one log lower than those in SH (10^6) at all dilutions. Onset as determined by PFU titers of brain homogenates generally corresponded to the dose given, i.e. infection within 24 hr occurred with any dose of $\geq 10^2$ PFU. We will attempt to obtain an LD₅₀ in both SM and SH in order to standardize challenge and protective doses of arenaviruses to be used in cross-protection studies.

In a continuing effort to develop rodent models, 55-day-old rats were inoculated IC with either the parent strain or 1 of 5 second-passage, adult mouse-brain isolates of Tacaribe virus strain 11573. All rats that became sick (20 of 24) initially had ruffled fur and hunched positions which progressed to prostration, hind-limb extension and paralysis, and eventual death. Onset of signs began at 10 to 11 days and continued throughout the 21-day observation period in those rats that survived. On day 14, one prostrate rat from each sample group was killed for examination by Pathology Division personnel. All showed nonsuppurative, multifocal, perivasicular meningoencephalitis, with neuronal necrosis and gliosis. Brain samples obtained at time of killing generally contained 10^3 PFU/ml of a 20% suspension. The rat encephalic model may be examined further in studies in which both parent and subpopulation isolates of strain 11573 will be administered by IC, IP, IM, and SC routes. Plasma and/or tissue samples will be obtained for subsequent serial passage when rats are killed for pathological examination at time of observable illness.

Attempts to develop a guinea pig model have generally been unsuccessful in that guinea pigs, inoculated with identical virus samples, described for rats, fail to show any observable effects attributable to virus infection. The one exception was a single guinea pig that had a transient, single hind-limb

paralysis on day 12 after IC challenge. We will not examine this model further.

Our studies indicate that varying degrees of protection are afforded by a single SC injection of viruses of the Tacaribe complex against lethal IC challenge of Tacaribe virus. The duration of protection could not be established, since mice exhibit an increasing resistance with age.

There is much we do not know about the cross-protection which appears to exist between viruses of the Tacaribe complex. A SC dose of Parana virus given 13 days prior to IC challenge with Tacaribe virus protected mice, whereas a similar dose of Parana virus administered 5 days prior to challenge did not protect. It would appear that direct viral interference did not occur if the interference was directly related to the total amount of virus present in the Parana virus dose. Further, we have been unable to demonstrate Parana virus by plaqueing samples of brain, kidney, spleen or plasma taken from mice that have received up to 10^5 PFU of virus SC. Therefore, direct interference by replication of the protecting virus does not appear to be a primary factor in cross-protection in this system. Conversely, protection may be a consequence of the presence of circulating CF and/or neutralizing antibodies which require 13 days to reach an effective level. We have been unable to demonstrate at time of challenge the presence of antibodies produced by Parana virus immunization in mice that are protected from Tacaribe virus challenge. Our inability to detect either Parana virus or antibodies in mice that are protected from Tacaribe virus challenge is perplexing, if one considered that a small protecting dose of virus would require considerable replication to cause either direct interference or reach sufficient antigenic mass to induce antibody formation. Neither appears to have occurred, yet protection existed. The possibility that protection is a type of cell-mediated immunity cannot be dismissed, since arenaviruses are known to elicit immunopathological changes traceable to cellular mechanisms.

The role of interferon in this system is also unknown. However, Tacaribe virus appears to be resistant to interferon induced in the mouse by poly I:C stimulation. When we treated mice with polyI:C and gave a protecting dose of Tacaribe virus, the induced interferon had no effect on the protecting virus as demonstrated by later survival of these mice after IC challenge with lethal Tacaribe virus. Moreover, the amount of interferon induced by Parana virus stimulation is not known, but based on the preceding observations with poly I:C, we assume that it would not be a primary factor in protection against Tacaribe virus challenge at normal levels.

In summary, cross-protection in mice, elicited by Parana virus against Tacaribe virus challenge, does not appear to be mediated by direct viral interference, interferon, or the presence of CF or neutralizing antibodies. If, in fact, the cross-protection observed in adult mice is the result of a Parana virus-stimulated cell-mediated mechanism, studies involving immunosuppression and adoptive transfer of immune spleen cells prior to Tacaribe virus challenge may become of interest in future studies on mechanisms of action.

Publication:

Rosato, R. R., E. L. Stephen, W. L. Pannier. 1976. Dose-response data for toxiferine dichloride in monkeys and guinea pigs. Toxicol. Appl. Pharmacol. 35:107-111.

LITERATURE CITED

1. Wildy, P. 1971. Classification and Nomenclature of Viruses. Monographs in Virology, Vol. 5. S. Karger, Basel p. 73.
2. Tauraso, N., and A. Shelokov. 1965. Protection against Junin virus by immunization with live Tacaribe virus. Proc. Soc. Exp. Biol. Med. 119:608-611.
3. Eddy, G. A. 1972 - date. USAMRIID Work Unit 834 03 405, Quarterly and Annual Reports.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION# DA 086429	2. DATE OF SUMMARY# 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SURVEY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SECY# U	6. WORK SECURITY# U	7. REGADING# NA	8. DISP'N INSTR'N NL	9b. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	9. LEVEL OF SUM A. WORK UNIT
10. NO./CODES: b. PRIMARY 62760A	PROGRAM ELEMENT	PROJECT NUMBER 3A762760A834		TASK AREA NUMBER 02	WORK UNIT NUMBER 804		
c. CONTRIBUTING <i>114(e)(f)</i>	CARDS 114(e)(f)						
11. TITLE (Pencils with Security Classification Code) (U) Controlled enzymatic and chemical alteration of microbial protein							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry							
13. START DATE 71 08	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house				
17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS 1.0	b. FUNDS (In thousands) 231.5		
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b. NUMBER: NA	c. TYPE: f. CUM. AMT.	20. PERFORMING ORGANIZATION					
d. KIND OF AWARD:		NAME: Pathology Division USAMRIID ADDRESS: Fort Detrick, MD 21701					
e. RESPONSIBLE DOG ORGANIZATION		PRINCIPAL INVESTIGATOR (Pencil SIGN IF U.S. Academic Institution) NAME: Spero, L. TELEPHONE: 301 663-7211 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME:					
f. RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		POC:DA					
21. GENERAL USE Foreign intelligence considered							
22. SUBJECT (Pencils with Security Classification Code) (U) Bacterial toxins; (U) Amino acids; (U) Enzymes; (U) Toxoids; (U) Vaccines; (U) Staphylococcus; (U) Laboratory animals; BW defense; (U) Military medicine							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Pencil individual paragraphs identified by number. Pencils test of each with Security Classification Code.) 23 (U) Prepare more effective immunogens against militarily important diseases in which proteins of bacterial and viral origin have significant biological effects. The immediate goal is the identification of the elements of the protein structures containing antigenic determinants and toxic sites. The enterotoxins produced by <i>Staphylococcus aureus</i> are now being studied. These toxins are potential agents for biological attack and are responsible for many outbreaks of food poisoning. 24 (U) Staphylococcal enterotoxin C (SEC) undergoes a rapid, limited digestion by trypsin. Studies are concerned with characterization of the fragments and their role in the serological, emetic, and mitogenic activity of the whole molecule. 25 (U) 75 07 - 76 06 - The product of tryptic digestion of SEC-1 in which 2 peptide bonds are hydrolyzed, SEC-1-T(2), has full emetic activity. Circular dichroic spectra indicate retention of major conformational features in the hydrolyzed form, but a reduction in conformational stability is observed. A comparison of the amino acid composition of the 6,500 MW amino terminal polypeptide and the adjacent 4,000 MW popypeptide of SEC-1-T(2) with the structurally equivalent segments of staphylococcal enterotoxin B (SEB) suggest considerable homology between SEB and SEC-1. The 22,000 and 6,500 MW polypeptides of SEC-1-T(2) recombine to form a particle with only minor alterations in physical conformation and full serologic and mitogenic activity. Antigenic determinants are demonstrable on both the 6,500 and 22,000 MW peptides. Both completely inhibit the precipitation of SEC-1-antiSEC-1 at equivalence. It is suggested that there are 2 determinants on the 22,000 polypeptide and 1 on the 6,500 polypeptide. Publications: J. Biol. Chem. 250:5026-5032, 1975. 251: in press, 1976. Infect. Immunol. 13:1018-1020, 1975; Fed. Proc. 35:1395, 1976							

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 804: Controlled Enzymatic and Chemical Alteration of Microbial Proteins

Background:

The staphylococcal enterotoxins are simple proteins elaborated by Staphylococcus aureus which cause emesis and diarrhea in a limited number of mammalian species. Several antigenically defined types, SEA, SEB, and SEC, have been isolated in very pure form; all consist of a single polypeptide chain with one disulfide bridge and have a MW of ~ 28,000. We have been investigating partial enzymatic breakdown as a means of separating and identifying antigenic and toxic sites. The more alkaline variant of SEC, called SEC₁, undergoes a limited digestion with trypsin yielding products having either a single peptide bond cleaved, SEC₁-T₁, or with 2 bonds cleaved, SEC₁-T₂. The first bond cleaved is in the disulfide loop and the second is on the amino terminal side of the loop at about position 57-58. SEC₁-T₂ behaves as a single particle. It possesses full antigenic activity and is nearly as potent a mitogen as the untreated SEC₁. SEC₁-T₂ can be separated into a 6,500 and a 22,000 MW polypeptide after unfolding in strong denaturants. The 6,500 MW polypeptide is not emetic but does have significant mitogenic activity. Its amino acid analysis shows similarity to the equivalent segment from SEB.

Progress:

The emetic activity of SEC₁-T₂ has been determined by IV assay over a 5-hr period in 3-kg rhesus monkeys; results are presented in Table I.

TABLE I. EMETIC ACTIVITY OF STAPHYLOCOCCAL ENTEROTOXIN C₁ AND ENTEROTOXIN C₁-T₂

DOSE μg/kg	NO. POSITIVE ^a /TOTAL (LATENT PERIOD, MIN)	
	Enterotoxin C ₁	Enterotoxin C ₁ -T ₂
1.0		3/4 (65, 111, 129)
0.3	3/4 (67, 81, 99)	3/4 (88, 92, 111)
0.1	3/4 (70, 74, 77)	3/4 (105, 157, 164)
0.03	2/4 (123, 176)	2/4 (122, 125)
0.01	0/4	

^aWith diarrhea and emesis/total tested.

It is clear that, within the limits of accuracy of a single assay, the derivative has full emetic potency. It is also noteworthy that SEC_1 is more potent than SEB. In 3 separate assays the IV ED_{50} was $\leq 0.03 \mu\text{g}/\text{kg}$. The reported ED_{50} for SEB is $0.1 \mu\text{g}/\text{kg}$.¹

It was noted earlier that $\text{SEC}_1\text{-T}_2$ behaves as a single molecular entity, and the emetic activity described above is the third different measure of biological activity indicating that no essential change in conformation occurs in going from SEC_1 to $\text{SEC}_1\text{-T}_2$. This conclusion is strengthened by the observation that the biological parameters reflect different aspect of surface structure.² Circular dichroism (CD), a sensitive physical measure of conformation, also indicates retention of major structural features. In comparative CD spectra of SEC_1 and $\text{SEC}_1\text{-T}_2$, no shifts in wavelength of the dichroic bands $> 1 \text{ nm}$ are observed. The folding of the peptide backbone is unchanged. A small decrease seen in the ellipticity of the major negative band at 217 nm is within the error of the technique. No significant change is evident either in the major near-UV negative bands at 279 and 285 nm. Real changes are seen in a positive band at 237 nm and in a negative band at 243 nm and may represent alterations in the environment of aromatic residues.

Conformational stability is affected. First-order kinetic plots of the unfolding in 4 M guanidine-HCl of SEC_1 and $\text{SEC}_1\text{-T}_2$ at 2 wavelengths were measured by CD. The extrema selected are the strongest band in the aromatic region at 285 nm and the band attributed to β -pleated sheet at 217 nm in the far-UV.³ At 285 nm the first-order reaction rate constants are $4.3 \times 10^{-3} \text{ min}^{-1}$ for SEC_1 and $8.8 \times 10^{-3} \text{ min}^{-1}$ for the derivative. At 217 nm the respective rate constants are $4.8 \times 10^{-3} \text{ min}^{-1}$ and $7.4 \times 10^{-3} \text{ min}^{-1}$. Thus the doubly nicked enterotoxin unfolds at about twice the rate of the parent material under these conditions.

SEC_1 has an amino terminal glutamic acid and a carboxyl terminal Gly residue.⁴ In the formation of $\text{SEC}_1\text{-T}_2$, 2 new amino terminal residues and 2 new carboxyl terminal residues are generated. Both of the latter are Lys residues. The new amino terminal residues are Val and aspartic acid (or asparagine) and both are on the 22,000 MW polypeptide. The 22,000 fragment can be separated after reduction and alkylation of the disulfide bridge into 4,000 and 19,000 MW polypeptides (all the MW are best estimates from amino acid analysis, see below). It was earlier assumed from the rate of appearance of the new N-terminal amino acids and by analogy with the structure of SEB that the smaller fragment was central and the larger, C-terminal. Direct chemical proof of this has now been obtained. An amino terminal Val was identified on the 19,000 fragment by the dansylation technique; moreover, Lys is liberated from the 4,000 fragment but not from the 19,000 by digestion with carboxypeptidase B. Thus the nicked peptide bond in the disulfide loop of $\text{SEC}_1\text{-T}_2$ is unequivocally Lys-Val and the order of peptides in $\text{SEC}_1\text{-T}_2$ is 6,500 MW-4,000 MW-19,000 MW.

An amino acid analysis of the 2 peptides of the 22,000 MW polypeptide was carried out and the MW computed from it by error minimization techniques.^{5,6} The results are shown in Table II.

TABLE II. AMINO ACID COMPOSITION OF THE TRYPTIC PEPTIDES FROM STAPHYLOCOCCAL ENTEROTOXIN C₁-T₂

AMINO ACID	RESIDUES/MOLECULE							ENTERO- TOXIN C ₁ ^a
	6500 MW (1)	22,000 MW (2)	4,000 MW (3)	19,000 MW (4)	SUM OF COL. 1 & 2	SUM OF COL. 1, 3, & 4		
Lys	8	25	5	18	33	31	31	31
His	3	4	0	3	7	6	6	6
Arg	0	3	0	3	3	3	3	3
Asp	10	31	7	31	41	48	43	
Thr	3	13	1	14	16	18	15	
Ser	4	10	3	9	14	16	15	
Glu	4	17	3	15	21	22	19	
Pro	3	4	0	4	7	7	6	
Gly	1	14	2	13	15	16	15	
Ala	4	5	1	4	9	9	7	
Cys/2	0	b	1 ^c	1 ^c		2	2	
Val	4	14	4	10	18	18	18	
Met	1	3	0	5	4	6	7	
Ile	2	9	0	10	11	12	10	
Leu	5	12	3	9	17	17	16	
Tyr	3	14	4	9	17	16	17	
Phe	2	9	1	9	11	12	10	
Trp							1	

^a

Recalculated from the data of Huang et al.⁴

^b

Not determined.

^c

Determined as S-carboxymethylcysteine.

The summation of all the fragments and a comparison with the composition of SEC₁ are also included in this table. It is apparent that reasonably good agreement was obtained. It was reported earlier that there was considerable similarity between the amino acid composition of the 6,500 MW polypeptide and the comparable segment of the amino acid sequence of SEB. A similar comparison is shown in Table III between the 4,000 MW peptide and its comparable segment in SEB i.e., residues 55-97.

TABLE III. COMPARISON OF THE AMINO ACID COMPOSITION OF THE 4000 MW POLYPEPTIDE OF ENTEROTOXIN C₁-T₂ WITH THE EQUIVALENT STRUCTURAL SEGMENT FROM ENTEROTOXIN B

AMINO ACID	RESIDUES/MOLECULES	
	4000 MW	Enterotoxin B 55-97
Lys	5	8
His	0	0
Arg	0	1
Asp	7	10
Thr	1	1
Ser	3	1
Glu	3	2
Pro	0	0
Gly	2	2
Ala	1	2
Cys/2	1	1
Val	4	4
Met	0	0
Ile	0	0
Leu	3	2
Tyr	4	6
Phe	1	3
Trp	a	0

^aNot determined.

Again good agreement is seen. These data suggest considerable homology between SEB and SEC₁.

We have previously shown that the 2 polypeptides of SEB-T readily reform to a single molecular entity with regeneration of full biological activity and physical properties. A similar result is obtained with SEC₁-T₂. When a solution of the 22,000 and 6,500 MW peptides in 6 M guanidine-HCl is dialyzed against PBS a single particle is obtained. It gives only a single peak on gel filtration with Sephadex G-50. It is a precipitating antigen and a comparison of its reactivity against rabbit anti-SEC₁ with the original derivative in the quantitative precipitin test shows no difference. Similarly mitogenic activity is fully restored. The circular dichroic spectrum of the recombined material is identical in the far-UV to SEC₁ and SEC₁-T₂. There are, however, changes in the near-UV. The increase in the positive extremum at 237 nm that occurs in going from SEC₁ to SEC₁-T₂ is magnified. There is no change in the band intensity at 243 nm. An additional perturbation, an increase in the negative ellipticity of the double band at 279 and 285 nm, is also observed. It appears that the recombination of the

22,000 and 6,500 polypeptides leads to a regeneration of the basic peptide folding of SEC₁ but some, possibly more flexible, elements of tertiary structure are somewhat distorted.

We previously reported that we were unable to demonstrate antigenic reactivity with either the 22,000 or the 6,500 polypeptide from SEC_{1-T₂}. The 6,500 material is soluble but the 22,000 fragment is insoluble in aqueous buffers. Although the latter could be solubilized with detergents, no activity results. Earlier reports of precipitation of antibody in Ouchterlony immunodiffusion have now been found to have resulted from contamination with SEC_{1-T₁}. We are now able to prepare stable solutions of the 22,000 polypeptide. The fragment in 6 M guanidine-HCl is concentrated to about 10 mg/ml in dialysis tubing embedded in Sephadex G-200. Careful addition of 1-μl quantities into a 10% solution of bovine serum albumin in phosphate buffer (0.05 M, pH 6.8) enables us to obtain concentrations up to 500 μg/ml. Neither of these solutions (in 6 M guanidine or 10% BSA) induces a precipitate in Ouchterlony immunodiffusion but both curve and shorten the precipitin line of SEC₁ with antibody indicating an inhibition of the precipitin reaction. Inhibition of precipitation in solution is also observed. An assay based on the development of turbidity at 570 nm of a system containing SEC₁ and a partially purified rabbit anti-SEC₁ at equivalence in a total volume of 505 μl is employed. Complete inhibition is obtained with a molar ratio of inhibitor/SEC₁ of 3.2:1 and 50% inhibition at a ratio of 1.4:1.

The technique of rapid dilution of a concentrated solution in 6 M guanadine also permits the demonstration of an antigenic determinant on the 6,500 MW polypeptide. Although this fragment is not nearly as potent as the 22,000 polypeptide, complete inhibition is obtained at a molar ratio of 25:1 and 50% inhibition at a molar ratio of 12.5:1. Obviously neither fragment has 3 operationally independent determinants since neither precipitates antibody. However, since at least 3 determinants are required in the intact enterotoxin molecule for matrix formation, we suggest the presence of 2 determinants on the larger fragment and 1 on the smaller. This is based on 3 considerations: (1) the small fragment shows no secondary structure by circular dichroism; (2) the size factor implies more surface reactive elements on the larger material; and (3) the larger fragment is an extraordinarily efficient inhibitor.

A manuscript describing the preparation and properties of SEC_{1-T₁} and SEC_{1-T₂} has been submitted for publication.

Presentation:

1. Spero, L., J. F. Metzger, B. Y. Griffin, and J. L. Middlebrook. Limited tryptic hydrolysis of staphylococcal enterotoxin C. Presented,

American Society of Biological Chemists, San Francisco, CA, 7 June 1976.
(Program and abstracts, no. 194). (Fed. Proc. 35:1395, 1976.)

Publications:

1. Spero, L., J. F. Metzger, J. R. Warren, and B. Y. Griffin. 1975. Biological activity and complementation of the two peptides of staphylococcal enterotoxin B formed by limited tryptic hydrolysis. *J. Biol. Chem.* 250: 5026-5032.
2. Spero, L., D. L. Leatherman, and W. H. Adler. 1975. Mitogenicity of formalinized toxoids of staphylococcal enterotoxin B. *Infect. Immunity* 12:1018-1020.
3. Spero, L., B. Y. Griffin, J. L. Middlebrook, and J. F. Metzger. 1976. Effect of single and double peptide bond scission by trypsin on the structure and activity of staphylococcal enterotoxin C. *J. Biol. Chem.* 251: in press.

LITERATURE CITED

1. Schantz, E. J., W. G. Roessler, J. Wagman, L. Spero, D. A. Dunnery, and M. S. Bergdoll. 1965. Purification of staphylococcal enterotoxin B. *Biochemistry* 4:1011-1016.
2. Spero, L., D. L. Leatherman, and W. H. Adler. 1975. Mitogenicity of formalinized toxoids of staphylococcal enterotoxin B. *Infect. Immunity* 12:1018-1020.
3. Greenfield, N., and G. D. Fasman. 1969. Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry* 8:4108-4116.
4. Huang, I-Y., T. Shih, C. R. Borja, R. M. Avena, and M. S. Bergdoll. 1967. Amino acid composition and terminal amino acids of staphylococcal enterotoxin C. *Biochemistry* 6:1480-1484.
5. Delaage, M. 1968. Sur la recherche du poids moléculaire le plus cohérent avec l'analyse des acides aminés d'une protéine. *Biochim. Biophys. Acta* 168:573-575.
6. Katz, E. P. 1968. Molecular weight determination from amino acid analysis data: a numerical method. *Anal. Biochem.* 25:417-431.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^b	2. DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL	
3. DATE PREV SUMMARY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SECY ^b U	6. WORK SECURITY ^b U	7. REGRADING ^b NA	8. DSB/PN INSTN ^b NL	9. SPECIFIC DATA-CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
				10. NO./CODES: ^b a. PRIMARY b. CONTRIBUTING c. 114(e)(f)	PROJECT NUMBER 3A762760A834	TASK AREA NUMBER 03	11. LEVEL OF SUM-A WORK UNIT 008
11. TITLE (Proceed with Security Classification Code) (U) Mathematical and computer applications in infectious disease research							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^b .003500 Clinical medicine; 004900 Defense; 009700 Mathematics and statistics							
13. START DATE 69 11	14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house			
17. CONTRACT/GRAANT a. DATES/EFFECTIVE: b. NUMBER: c. TYPE: d. KIND OF AWARD:		EXPIRATION: NA	18. RESOURCES ESTIMATE FISCAL YEAR 76 77	19. PROFESSIONAL MAN YRS CURRENT 0.5 1.0	20. FUNDS (in thousands) 32 78		
21. RESPONSIBLE DOD ORGANIZATION NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701			22. PERFORMING ORGANIZATION NAME: Physical Sciences Division USAMRIID ADDRESS: Fort Detrick, MD 21701				
23. RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833			24. PRINCIPAL INVESTIGATOR (Provide NAME // U.S. Academic Institution) NAME: Higbee, G. A. TELEPHONE: 301 663-2640 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: Powers, T. J. NAME:				
25. KEYWORDS (Proceed EACH with Security Classification Code) (U) BW defense; (U) Military medicine; (U) Diagnosis; (U) Computers; (U) Medicine; (U) Statistics							
26. TECHNICAL OBJECTIVE, ^b 26. APPROACH, 26. PROGRESS (Provide individual paragraphs identified by number. Proceed each with Security Classification Code.) 23 (U) Develop and apply techniques for utilization of computers, statistics and mathematics to process and interpret biomedical data in a research program for medical defense against BW agents, emphasizing diagnostic, therapeutic and immunoprophylactic studies.							
24 (U) Theories and disciplines of numerical analysis, differential equations, statistical tests of hypotheses, experimental design, information storage and retrieval, and pattern recognition are utilized to analyze and interpret data gathered by investigators.							
25 (U) 75 07 - 76 06 - The installation of a computer terminal has done much to enhance the effectiveness of this work unit. The work begin in previous years has been completed, or continued either under this work unit or its associated Technical Support Plan (TSP-04). Research efforts have been concentrated in the areas of biochemical indices of infection, kinetic modeling of physiological systems, and in statistical consultation							
Publication: J. Appl. Physiol. 40:101-104, 1976.							

^aAvailable to contractors upon originator's approval.

DD FORM 1498 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

G U S P O: 1974-840-843/8691

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Aspects of Biological Agents (U)

Task No. 3A762760A834 03: Laboratory Identification of Biological Agents

Work Unit No. 834 03 008: Mathematical and Computer Applications in
Infectious Disease Research

Background:

The objective of effective utilization of computers, statistics, and mathematics in handling data generated within the Institute is pursued in 3 main areas: early diagnosis of infection, mathematical modeling and analysis of physiological systems, and consultation. Work is generally performed in conjunction with many other work units, often allowing a single technique or solution to be applied to similar problems.

During the past year the newly-installed Data 100 computer terminal connected to WRAIR's CDC 3500 computer has provided the majority of computer support needed by USAMRIID for the processing of scientific data. The availability of a terminal has improved turnaround for computer jobs and increased the effectiveness of the Computer Science Office in responding to new programming requests by investigators.

The options for computerized statistical analysis have also been expanded by making it possible to access the BMD Biomedical Computer Programs, developed at UCLA, from WRAIR's computer via our terminal. A Univac 1108 emulation software package was acquired that permits our use of the Univac 1108 computer at the National Bureau of Standards (NBS) from our remote terminal. NBS subscribes to the computer subroutine libraries in mathematics and statistics (IMSL) developed and maintained by International Mathematical and Statistical Libraries, Inc. As a remote terminal user of NBS, the Institute now has access to this excellent library of statistical procedures. Remote terminal access to NBS has also facilitated the kinetic modeling of physiological systems using the Simulation Analysis and Modeling (SAAM) program.

Progress:

Work is continuing in the area of determining if diagnostic profiles of biochemical changes can be developed which are characteristic of specific infectious processes. Biochemical analyses on the available blood samples are now complete, resulting in a data base of 393 samples from West Virginia University, 317 samples from the University of Maryland, and USAMRIID analyses of 795 serum samples. Results of these analyses have been keypunched and stored on computer disc files.

Computer programs are now operational which sort and analyze the data for possible biochemical indices of infection. The sorting subroutines sort the data on 1 or 2 of 12 possible personal identification parameters and patient categories. The data can be further sorted on any 1, 2, or 3 diagnoses. After all sorting is completed by the computer, subroutines perform statistical analyses for any of 3 possible conditions: (1) Control vs. Ill, (2) Illness A vs. Illness B, or (3) Illness A vs. Illness A for 2 different personal parameters. Work is continuing in this area to determine the most appropriate statistical tests for the various biochemical indices. Parametric and nonparametric statistical procedures will be used according to the nature of the population distribution of the measured variables. Statistical tests will be specified and computer programs written for these tests.

A limited study on the affects of aging on 6 serum proteins, 2 amino acids, and 3 trace metals for viral and bacterial infections was carried out using a portion of the data base. The trace metal responses were found to be the same for each of the 4 age groups and for both infections considered. Zn decreased slightly from controls. Serum Fe decreased significantly, and Cu increased significantly.

Efforts in kinetic modeling of physiological systems have been in 2 areas: (1) models of viral dynamics and (2) pharmacokinetics. By deletion of one compartment in the basic viral compartmental model¹ corresponding to host defenses, a good fit was achieved to data describing virus replication with no host defenses, i.e., virus levels increasing exponentially until reaching a maximum value, after which they remained elevated and fairly constant.

The basic compartmental model used for analysis of influenza population dynamics has been used to describe unadapted Sendai virus dynamics. Work is continuing in correlating the magnitudes of various compartmental parameters with each other, in correlating specific compartments of the model with physiological processes of the immune system, and in determining the best time-interrupt at which to stop the exponential viral growth pattern and initiate the action of compartments representing saturation of viral growth and host defenses.

The SAAM program was also used to fit pharmacokinetic data to the sum of 2 decaying exponentials for drug (Ribavirin) distribution in 5 tissues of mice, inoculated IP. For aerosol exposures, the responses were not satisfactorily fit by sums of exponentials. Additional pharmacokinetic studies will be analyzed upon completion of planned experiments in Aerobiology Division.

A computer program to calculate protein molecular weight from amino acid residues was developed, based on the techniques of Delaage.² The Computer Science Office was subsequently requested to develop a second program for MW determination, using a slightly different procedure developed by Katz.³ The Katz version has been completed and gives results close to those by the Delaage technique. The "best" MW for a given input set of amino acid residues is that which results in a minimum sum of squares of the differences between the analytical composition of an amino acid and its nearest integer for a trial MW. The Katz program generally gives a smoother changing sum of squares than the Dellage procedures. A plot subroutine has been added to both computer programs to allow visual inspection of the changing sum of squares as the trial MW changes.

Consultations were held with investigators of all divisions of the Institute to develop solutions to short-term computational or statistical problems.

In the process of solving data analysis problems given to the Computer Science Office, 2 generalized computer programs were recently developed that will have application to the projects of numerous investigators throughout the Institute. The specific computer programs were: (1) two-way analysis of variance of repeated measurements, and (2) comparison of proportions from many samples by the partitioning of $2 \times c$ contingency tables for multiple independent comparisons.

Publications:

Liu, C. T., and G. A. Higbee. 1976. Determination of body surface area in the rhesus monkey. *J. Appl. Physiol.* 40:101-104.

LITERATURE CITED

1. Larson, E. W., J. W. Dominick, A. H. Rowberg, and G. A. Higbee. 1976. Influenza virus population dynamics in the respiratory tract of experimentally infected mice. *Infect. Immun.* 13:438-447.
2. Delaage, M. 1968. Sur la recherche du poids moléculaire le plus cohérent avec l'analyse des acides aminés d'une protéine. *Biochim. Biophys. Acta* 168:573-575.
3. Katz, E. P. 1968. Molecular weight determination from amino acid analysis data: a numerical method. *Anal. Biochem.* 25:417-431.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^b DA OC6414	2. DATE OF SUMMARY ^b 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUMMARY 75 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCY ^b U	6. WORK SECURITY ^b U	7. REGRADING ^b NA	8. DSBPN INSTN ^b NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES ^b	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER	
a. PRIMARY b. CONTRIBUTING	62760A	3A762760A834		03	011	
c. EQUIPMENT d. EQUIPMENT	CARDS 114(e)(f)					
11. TITLE (Pencode with Security Classification Code) (U) Chemical mediators of infection of military medical importance						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^b 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry						
13. START DATE 71 02	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-house		
17. CONTRACT/GRAANT	18. RESOURCES ESTIMATE FISCAL YEAR		19. PROFESSIONAL MAN YRS CURRENt		20. FUNDS (in thousands)	
a. DATES/EFFECTIVE: b. NUMBER: c. TYPE: d. KIND OF AWARD:	EXPIRATION: NA	PRECEONS 76	1.0	163	77	1.0
e. CUM. AMT.				201		
21. RESPONSIBLE DOD ORGANIZATION NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701	22. PERFORMING ORGANIZATION NAME: Physical Sciences Division ADDRESS: USAMRIID Fort Detrick, MD 21701					
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833	PRINCIPAL INVESTIGATOR (Punish DOD or U.S. Academic Institution) NAME: Mapes, C. A. TELEPHONE: 301 663-7181 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME:					
23. GENERAL USE Foreign intelligence considered	POC:DA					
24. KEY WORDS (Pencode with Security Classification Code) (U) Prostaglandins; (U) Lipids (U) BW defense; (U) Military medicine; (U) Phagocytosis; (U) Leukocytic mediators; (U) Host metabolism; (U) Bioassay; (U) Endotoxin tolerance						
25. TECHNICAL OBJECTIVE ^b & APPROACH. 26. PROGRESS (Pencode individual paragraphs identified by number. Pencode last of each with Security Classification Code.)						
23 (U) First, isolate and identify mediators that regulate the host's metabolic responses to infection. Second, knowledge gained through identification studies will be applied to detection of specific compounds or their precursors which may arise during the asymptomatic stage of viral or bacterial infections. Success of these detection studies is applicable to screening military units suspected of exposure to natural or BW agents. Third, attempt development of prophylactic or therapeutic method for controlling the mediators of infection, since medical treatment is an essential element in maintaining the military strength of those units exposed to or infected by natural or BW agents.						
24 (U) Study the effects of endogenous mediator(s) on various aspects of host metabolism in order to elucidate the mechanisms involved during infectious illness.						
25 (U) 75 07 - 76 06 - Crude PMN-derived preparations have been separated into 5 fractions, each containing substance(s) that can induce a specific metabolic alteration. Substances present in these fractions are: endogenous pyrogen, amino acid-fluxing activity; zinc-depressing activity; and 2 leukopoietic factors which can independently increase total peripheral neutrophils.						
A new area of research has implicated prostaglandin biosynthesis in production of PMN-derived mediators. More extensive studies tend to support the hypothesis that mediator activities, excluding pyrogenicity, are due to either prostaglandin or prostaglandin-protein complexes.						
Other areas investigated include: endotoxin tolerance in rats and rabbits; production of mediators by PMN homogenates; and the effect of several compounds on production of mediators. These compounds include: protein synthesis inhibitors, reduced glutathione, metal ions, and Solucortex (trade name).						
Publications: 1976 Army Science Conf. Proc. II:405-419, 1976; Am. J. Physiol. 231: in press, 1976; Prostaglandin in press 1976						

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 03: Laboratory Identification of Biological Agents

Work Unit No. 834 03 011: Chemical Mediators of Infection of Military Medical Importance

Background:

The crude material obtained from glycogen-stimulated rabbit peritoneal exudate polymorphonuclear leukocytes (PMN) alters several biological parameters when administered to laboratory animals. Metabolic alterations observed in rats include: decreased serum Zn and Fe concentrations concomitant with their increased concentrations in liver; increased concentrations of serum Cu; ceruloplasmin, α_1 - and α_2 -acute-phase globulins; increased levels of peripheral neutrophils, hepatic amino acid uptake; increased RNA synthesis; and decreased portal vein glucose concentrations accompanied by increased concentrations of glucagon and insulin.¹⁻⁴ The crude PMN-derived material also causes a febrile response when administered to rabbits.⁵ These metabolic and physiologic alterations, characteristic of host responses observed during infectious and inflammatory processes, are attributed to the presence of one or more mediators. Classically, the mediator of febrile response has been termed endogenous pyrogen (EP) while leukocytic endogenous mediator (LEM) has been used as a general term to denote the substance(s) mediating multiple inflammatory responses observed in rats. Although the latter substance has not been demonstrated to differ from EP, it has been postulated that LEM may be an intermediate in stimulation and regulation of host responses during infections, endotoxemia and acute inflammatory stresses.

Progress:

Differentiation between LEM and EP. It has been assumed that LEM and EP are either the same or closely related molecules since they are produced concomitantly from stimulated PMN, are reported affected in the same manner by many chemical reagents, and have not been separated by fractionation techniques thus far employed. However, we noticed several subtle differences in the characteristics of LEM and EP. Comparative experiments utilizing properties characteristic of EP, therefore, were designed in an attempt to determine whether LEM and EP were the same molecule. Evidence supporting the differentiation between LEM and EP include: (a) physical separation of EP from one or more mediators that induce metabolic alterations attributed to LEM; (b) production of LEM activities by stimulated PMN in the absence of detectable pyrogenic activity; and (c) differences between "release" of

EP and LEM from stimulated PMN in the presence of morphine, a partial-nitrogen atmosphere, or K⁺.

Physical separation of EP and LEM. Literature reports indicate that pyrogen purification is plagued by continuous loss of biological activity due to its nonspecific binding to glass. Therefore, 75-150-μm glass beads were tested as a possible affinity adsorbent for leukocyte mediators; ~ 80% of the total protein in a mediator preparation bound to glass. There was no detectable pyrogenic activity in the unbound material in comparison with the untreated mediator preparation. Since attempted elution of pyrogenic activity from the glass was unsuccessful, the experimental conditions were simulated to insure that they had no detrimental effect on activity. A statistically insignificant decrement of 0.2°C fever in this control, indicated that pyrogenic activity was bound to the glass beads rather than inactivated by the experimental conditions.

In contrast, there was no detectable loss of Zn-depressing neutrophil-enhancing, or amino acid-fluxing activities after binding to glass. This statement is based on analyses of 3-point dose-response curves comparing LEM activities before and after adsorption to glass. It should be noted, however, that a 10% loss of the initial activity would not be detected due to the experimental error inherent in the rat bioassay.

Production of LEM activities by stimulated PMN in the absence of detectable pyrogenic activity. During the weekly production and analyses of mediator preparations, it was fortuitously noted that not all preparations contained detectable pyrogenic activity although they were not unusual with respect to the LEM activities bioassayed. The arbitrary criteria for evaluating an active mediator preparation as assigned by this laboratory are: (a) in comparison to the heat-inactivated preparation, a 1.0-ml test sample will cause a 40% depression of plasma Zn, a 1.5-fold increase in hepatic amino acid uptake and a 2-fold elevation in peripheral blood neutrophils 5 hr after IP administration to rats; and (b) a 50-μl aliquot, diluted to 1.0 ml with pyrogen-free saline, will induce 0.5°C fever in rabbits 45-60 min following IV administration.

Comparative analyses of several preparations showed that mediators prepared between late July and late September did not induce statistically significant rabbit fevers, although they induced plasma Zn depression, hepatic amino acid uptake, and elevation of total blood neutrophils in the rat. These data further suggest a seasonal variation in pyrogen production, a speculation that has since been confirmed by personal communication with Dr. Patrick Murphy (Johns-Hopkins University, Baltimore, MD).

Differences in production of LEM and EP in the presence of various compounds. The effect of various compounds on in vitro production of mediators by stimulated PMN has been studied in several experiments. These experiments

have extended other observations which indicate that EP and LEM are separate molecular entities.

Stimulated PMN incubated in the presence of 2 mM morphine did not produce pyrogenic activity. Conversely, morphine had no detrimental effect on production of substances mediating plasma Zn depression, hepatic amino acid uptake, or increases of total blood neutrophils. Crude mediators prepared in the presence of the morphine, however, induced significant increases of total blood neutrophils in comparison to control incubations.

The effect of a partial-nitrogen atmosphere on mediator production also demonstrated differences between LEM and pyrogenic activities. Flushing a stimulated-PMN system with nitrogen (3 ml/min for 10 min) prior to incubation had no detrimental effect on production of substances mediating plasma Zn depression, hepatic amino acid uptake, or elevation of total blood neutrophils. EP, however, was not produced in the presence of a partial-nitrogen atmosphere.

The inhibitory effect of K^+ on "release" of EP was confirmatory of previous reports demonstrating 90% inhibition of EP release by 3-5 mM K^+ . The complete inhibition of EP release by 5 mM K^+ in our studies might be explained by the fact that our preparations contained 3 mM K^+ and 1-3 mg/100 ml Ca^{2+} prior to addition of electrolytes. Unlike EP, LEM release was detectably inhibited only at concentrations > 5 mM K^+ . Release of plasma Zn-depressing activity was inhibited by 10 mM K^+ , while release of amino acid-fluxing and neutrophile-enhancing activities were inhibited by nonphysiological K^+ concentrations of > 20 mM.

Separation of multiple leukocytic endogenous mediators. Having demonstrated that crude mediator preparations contain EP as a separate molecular entity, it became of interest to determine whether LEM itself was composed of multiple components.

Separation of an amino acid-fluxing activity. Adaptation of a butanol-methanol (BuOH-MeOH) fractionation technique for mediator purification was as follows: (All organic solvents and mediator preparations were chilled at 4°C prior to use and were maintained at 4°C throughout the experiment.) BuOH was added at a rate of 0.1 ml/min to a slowly stirred preparation until its final concentration reached 20% (v/v). The mixture was stirred an additional 15 min, then separated into a biphasic solution by centrifugation at 900 g for 10 min. After decanting the upper butanolic phase, MeOH was added at a rate of 0.2 ml/min to the slowly stirred interfacial and aqueous phases until the mixture was 60% methanol (v/v). This solution was allowed to stand 30 min prior to centrifugation at 16,000 g for 30 min to remove precipitated protein. Organic solvents were removed by dialysis against distilled water or physiological saline prior to bioassay. Alternatively, only the BuOH extraction step was employed for separation of mediators.

Bioassays of the BuOH-soluble, MeOH-soluble and -insoluble fractions resulted in the following information: (a) all of the detectable amino acid-fluxing activity was recovered in the BuOH-soluble fraction; (b) all of the detectable Zn-depressing activity was recovered in the MeOH-soluble fraction; (c) most of the neutrophile releasing activity initially detected in the mediator preparation was recovered in the MeOH-soluble fraction; in addition, a small amount of this activity was detectable in the BuOH-soluble fraction; and (d) the MeOH-insoluble fraction contained no detectable activity.

Additional studies demonstrated that precipitation of contaminating protein with MeOH was not an efficient step for fractionation or purification, since its addition to a final concentration of 60% resulted in a large sample volume which required removal of organic solvents and concentration prior to bioassay. A substantial or complete loss of mediator activity was common during this process. Therefore, the alternative of utilizing only BuOH extraction was selected.

Extraction of several mediator preparations with BuOH indicated that only the amino acid-fluxing activity is in soluble phase. The presence of neutrophil-releasing activity is detectable in the butanolic extract only when it has been contaminated with the insoluble material at the BuOH-water interface.

Analyses of protein and pyrogenic activity demonstrated that ~ 20% of the protein in a mediator preparation is BuOH-soluble. When this extraction is followed by MeOH treatment, an additional 14% of the initial protein remains in the supernatant, while the remaining protein is precipitated in a biologically inactive form.

Pyrogenic activity is always associated with the BuOH-insoluble fraction. It is, however, soluble in 60% MeOH. Compilation of the butanol-methanol data suggests that hepatic amino acid-fluxing activity is due to a molecular entity that does not induce fever, elevates total blood neutrophils, or depresses plasma Zn.

Separation of a Zn-depressing activity. Amberlite CG-50 (2.0 gm) was added to 50 ml of a mediator preparation. This mixture was stirred slowly for 30 min at 4°C, then the resin was sedimented by centrifugation at 900 g for 15 min at 4°C. An additional 2.0-gm portion of Amberlite CG-50 was added to the 900 g supernatant solution and the above procedure was repeated. The supernatants were combined and the volume was adjusted to 50 ml by addition of physiological saline. An attempt was made to recover mediator activities from the resin by sequential elution with 25.0-ml portions of 0.75 M NaCl.

All of the plasma Zn-depressing activity originally detected in the mediator preparation was bound to Amberlite CG-50 and was not eluted with 0.75 M NaCl. Conversely, none of the detectable amino acid-fluxing activity was bound to the resin. Approximately one-half of the neutrophil-releasing activity in the crude preparation bound to the resin and was eluted. Endogenous pyrogen, if present in the preparation, also was bound irreversibly to Amberlite CG-50. (About 90% of the crude mediator protein bound to Amberlite. An additional 0.8% was eluted with NaCl).

Additional studies utilizing a preparation that had no detectable pyrogenic activity revealed that batchwise Amberlite CG-50 chromatography could be performed with a dialyzed, BuOH-insoluble fraction. This fraction gave the same experimental results as the crude LEM preparation. All of the detectable Zn-depressing activity was irreversibly bound to the resin, while all of the detectable neutrophil-releasing activity could be accounted for in the unbound and eluted fractions. It thus became apparent that plasma Zn-depressing activity differed from neutrophil-releasing activity and that both differed from either pyrogenic or amino acid-fluxing activities.

Separation of leukopoietic factors in mediator preparations. The increase in total blood neutrophils observed after administration of crude PMN-derived mediators appears to result from 1 of 2 peripheral alterations occurring either independently or simultaneously. One alteration consists of an increase in the total number of circulating leukocytes without any detectable alteration in the differential cell count. The other results from an increase in the percentage of neutrophils without a detectable elevation in the total number of circulating leukocytes.

The possibility that 2 neutrophil-enhancing factors are produced by stimulated PMN was suggested by bioassay results obtained with numerous mediator preparations. Approximately 20% of the crude preparations caused an increase in the total number of circulating leukocytes; whereas, the same preparations consistently increased the differential neutrophil count. Since 2 similar activities, termed leukocyte- and neutrophil-inducing factors, had been separated from the plasma of rats subjected to leukocytaphoresis an attempt was made to separate PMN-derived neutrophil-enhancing activities using a similar procedure.

Solid $(\text{NH}_4)_2\text{SO}_4$ was added slowly to a stirred mediator preparation (50 ml), maintained at 4°C, until the solution was 40% saturated. After 30 min additional stirring, the precipitated protein was collected by centrifugation at 16,000 g for 30 min at 4°C. The 40% $(\text{NH}_4)_2\text{SO}_4$ supernatant and the precipitate (solubilized in 30 ml physiological saline) were each dialyzed overnight against saline. During the dialysis period, a rust-brown material sedimented from the solubilized 40% precipitate. This material was collected by centrifugation at 1200 g and resuspended in saline. All fractions were

adjusted to a final volume of 50 ml prior to bioassay.

The insoluble material sedimenting from the resuspended precipitate was the only fraction that contained detectable activity for causing an elevation in the total number of circulating leukocytes (NEF₁). Plasma Zn-depressing activity, hepatic amino acid-fluxing activity and a second leukopoietic factor that enhanced the neutrophil differential cell count (NEF₂) were recovered in the 40% supernatant. Pyrogenic activity, if present, also was recovered in the supernatant. Although NEF₁ and NEF₂ possess activities characteristic of other leukopoietic factors, sufficient characterization studies have not been completed to enable any comparison of chemical or physical properties. In addition, it is important to note that neither leukopoietic activity can be attributed to nonspecific responses induced by administration of heparin or foreign protein.

Summation of the data presented indicates that crude mediator preparations can be separated into 5 fractions, each containing a substance that can induce a metabolic or physiologic alteration characteristic of the host inflammatory response.

Interrelationship of PMN-derived mediators and prostaglandins. Prostaglandins are recognized as mediators of certain features of the inflammatory response, particularly in the manifestation of tissue injury. More recently they also have been shown to be present in some inflammatory exudates and to be chemotactic for PMN in an in vitro system. In addition, prostaglandins, some of the most pyrogenic naturally-occurring substances, have been shown to play an intermediary role in the induction of febrile responses which are mediated by the PMN-derived substance, EP. The prostaglandins (PG), however, previously had not been demonstrated to affect either the production or action of other PMN-derived mediators.

Experimental results obtained in this laboratory lead to the hypothesis that PG biosynthesis may have a role in the formation or activation of PMN-derived substances which mediate increases in total blood neutrophils, hepatic amino acid uptake, and depression of plasma Zn. Data supporting this hypothesis include: (a) prostaglandin synthetase inhibitors (23 μ M indomethacin or 93 μ M aspirin) caused 100% inhibition of mediator production; whereas, the control drug morphine (2 mM) had no detrimental effect on production of substances mediating plasma Zn-depressing, hepatic amino acid uptake, or increases of total blood neutrophils; (b) addition of 2 μ M PGE to stimulated PMN resulted in \sim a 2-fold increase in hepatic amino acid-fluxing activity without affecting plasma Zn-depressing or neutrophil-enhancing activities; (c) addition of 2 μ M PGF to stimulated PMN resulted in \sim a 2-fold increase in plasma Zn-depressing activity without affecting amino acid-fluxing or neutrophil-enhancing activities; and (d) addition of dibutyryl analogs of cyclic AMP or cyclic GMP (2 mM) to stimulated PMN caused no

significant alteration in production of mediators. These results indicate that PG do not influence mediator production through modulation of intracellular cyclic nucleotide levels.

Possible role of prostaglandins in mediating host inflammatory responses.

Although studies indicate that PG enhance mediator activities released in their presence, they do not answer the question of whether LEM is prostaglandin in nature. Results of several experiments suggest that either PG or PG-protein complexes may mediate some of the inflammatory responses observed following LEM administration. The following experimental observations support this conclusion: (1) Active mediator preparations contain PGE₁, PGE₂, PGF_{2α}, PGB, and PGA; whereas, prostaglandins have not been detected in inactive mediator preparations. (2) A biologically active fraction which contains protein and PG-like material has been isolated from crude LEM. When the association between PG and protein is disrupted, neither fraction has detectable biological activity. (3) Addition of 5 ng/ml PGF to an inactive LEM preparation followed by a 2 hr incubation at 37°C under nitrogen resulted in a preparation that induced 50% depression of plasma Zn in comparison to controls incubated under nitrogen in the absence of PG. Analogous procedures utilizing PGE resulted in a 2-fold elevation of amino acid-fluxing activity. Treatment of the inactive preparation with nitrogen or addition of PG without an incubation period did not render the preparation active. (4) Stimulated PMN will utilize [¹⁻¹⁴C]arachidonic acid for PG synthesis. Column chromatography of the labeled-mediator preparation of Sephadex G-50 showed that ~ 20% of the protein had an apparent MW of 20,000-30,000. The labeled-PG were associated exclusively with this protein fraction. The remaining protein had an apparent MW of < 3,000. There was no labeled PG associated with this fraction. These results indicate that the PG in a mediator preparation are associated with a particular protein fraction. (5) Addition of 15-prostaglandin dehydrogenase (PGDH) to a stimulated-PMN preparation after a given incubation period resulted in destruction of mediator activity. Since PGDH initiates destruction of PG, it was concluded that PG are required for mediator activity.

Preparation of PMN-derived mediators. Classically, it has been assumed that mediators are produced by an intact cellular system. It further was assumed that the cells remained intact during an incubation period, with various substances being released through the membrane. However, it has been demonstrated that stimulated-PMN homogenates produce mediators that do not differ in heat-lability or biological activity from those prepared from an equal concentration of initially intact, stimulated PMN. Electron microscopy of stimulated rabbit peritoneal PMN before and after incubation further support the concept that intact cells are not required for mediator production. Following 2 hr incubation in saline, initially intact PMN do not appear different from those homogenized prior to incubation.

Effect of various compounds on mediator production. The effect of several compounds was studied in an attempt to gain further insight into mediator production. The effect of each compound was tested during the 2 hr in vitro incubation for production of LEM. The activities assayed were plasma Zn depression, total neutrophil elevation, and hepatic amino acid uptake. The compounds tested, their effect, and some of the possible interpretations are listed:

1. Inhibitors of protein synthesis (cyclohexamide, puromycin and actinomycin D): Actinomycin D has no effect on production of leukocyte mediators, whereas, cyclohexamide and puromycin slightly enhance mediator production. These results indicate that protein synthesis does not occur during the in vitro incubation period, and are consistent with studies in the PG field which show enhanced PG production in the presence of cyclohexamide and puromycin due to inhibited synthesis of PG dehydrogenase. Actinomycin D has no effect on in vitro PG synthesis.

2. Glutathione, reduced: GSH enhances mediator production if added to the incubation 5 min prior to its termination. Addition of GSH at the beginning of the 2 hr incubation period suppresses mediator production. Although there are many possible interpretations, these findings are again consistent with PG synthesis. Utilizing optimal conditions of PG synthesis, GSH can cause a 4-fold elevation in the formation of some PG. This compound is not incubated in PG-synthesizing systems for extended periods of time, since it causes rapid synthesis of prostaglandins making them available for degradation.

3. Metal ions (Zn and Cu): The effect of these ions on mediator production was tested initially due to differences in the metal ion concentration of mediators prepared by Merrell-National Laboratories and USAMRIID. The effect of Zn was also of interest due to its reported action on PMN; it was inhibitory to mediator production; whereas, low concentrations of Cu were stimulatory. Cu is a cofactor in synthesis of PGF. The effect of Zn on PG synthesis is unknown, however, other heavy metals (Au, Ag, Hg, and Cd) are inhibitory to PG synthesis. Other possible effects of Zn, including its reported effect on biological membrane stabilization, have not been conclusively ruled out. However, its effects on lysosomal stabilization cannot be correlated with mediator production. Other compounds (e.g., morphine) showing similar effects on lysosomal stabilization do not inhibit mediator production.

4. Solu-Cortef (hydrocortisone sodium succinate): This compound was tested because of its reported inhibitory effect on EP production. It has no effect on production of other leukocyte mediators.

Endotoxin tolerance in rats and rabbits. Administration of endotoxin to rats engenders the same metabolic responses as injection of LEM, making it imperative that one be able to prove that activity is due to a leukocytic-derived product rather than a bacterial contaminant. The use of endotoxin-tolerant animals has been strongly emphasized as a tool for this purpose. Thus, it became important to confirm reports concerning induction of endotoxin tolerance in rats, as well as to determine whether an animal rendered tolerant to a specific endotoxin would be tolerant to all endotoxins.

Rats were given 7 daily IP injections of 10 µg Escherichia coli endotoxin 0127:B8. On day 7, each rat was given 1.0 µCi [¹⁴C]AIB/100 gm body wt. Individual groups of rats were challenged with the following endotoxins on day 8: 1, 10 and 100 µg E. coli 0127:B8; 10 µg E. coli 0111:B4; 10 µg Salmonella enteritidis; 10 µg Salmonella typhimurium; and a mediator preparation containing heat-stable activity. Control animals, given 7 daily injections of saline, also were tested for biological responses to the challenge endotoxins.

"Tolerant" rats showed no depression of plasma Zn after administration of either 1 or 10 µg E. coli endotoxin 0127:B8, but responded to the 100 µg dose of endotoxin. In contrast, rats were not tolerant to this endotoxin with respect to neutrophil release or hepatic amino acid uptake. Likewise, there was no tolerance to any biological response following administration of any of the other endotoxins or the heat-stable LEM preparation. These results were confirmed and extended using S. typhimurium and S. enteritidis endotoxin to induce tolerance.

The same endotoxins were administered to rabbits following published immunization schedules for rendering them endotoxin-refractory. In each case, the rabbits were endotoxin-tolerant with respect to febrile responses; however, they were not tolerant with respect to neutrophil release or plasma Zn depression. Hepatic amino acid uptake was not assessed in endotoxin-tolerant rabbits.

The inability to render rats endotoxin-tolerant necessitated investigation of possible alternatives for dealing with heat-stable mediator preparations since they are assumed to be endotoxin contaminated. The following experimental results have given a practical solution to this problem: (a) acid-washed charcoal (1.0 mg/ml) consistently renders LEM preparations heat labile without diminishing their biological activity; (b) rabbit febrile responses to endotoxin (2 ng/kg body wt) are easily recognized by the prolonged fever and characteristic shape of the temperature curve; and (c) 1 µg of endotoxin is required to induce "LEM-like" responses in a 120-180-gm male rat. The logical deductions from this information are: (a) removal of heat stable material from mediator preparations enables an assessment of their biological activity; and (b) if a given preparation does not induce an endotoxin fever in rabbits, its biological activity in rats cannot be attributed to endotoxin.

Publications:

1. Mapes, C. A., and P. Z. Sobocinski. Differentiation between endogenous pyrogen and leukocytic endogenous mediator. Am. J. Physiol., in press.
2. Mapes, C. A., and P. Z. Sobocinski. Multiple leukocytic factors that induce reactions characteristic of the inflammatory response. 1976 Army Science Conference Proceedings, in press.

LITERATURE CITED

1. Beisel, W. R., and R. S. Pekarek. 1972. Acute stress and trace element metabolism. Int. Rev. Neurobiol. Suppl. 1:53-82.
2. Kampschmidt, R. F., R. D. Long, and H. F. Upchurch. 1972. Neutrophil releasing activity in rats injected with endogenous pyrogen. Proc. Soc. Exp. Biol. Med. 139:1224-1226.
3. Wannemacher, R. W., Jr. Dec. 1972. Current concepts of the regulation of early metabolic changes. p. 113-121. In Commission on Epidemiological Survey, Annual Report FY 1972, to the Armed Forces Epidemiological Board, Washington, D.C.
4. George, D. T., F. B. Abeles, and M. C. Powanda. 1975. Alterations in plasma glucose, insulin and glucagon induced by a leukocyte derived factor(s). Clin. Res. 23:320A.
5. King, M. K., and W. B. Wood, Jr. 1958. Studies on the pathogenesis of fever. III. The leukocytic origin of endogenous pyrogen in acute inflammatory exudates. J. Exp. Med. 107:279-289.

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10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER	
a. PRIMARY	62760A	3A762760A834		03	103	
b. CONTRIBUTING						
c. 11111111	CARDS 114(e)(f)					
11. TITLE (Puruse each with Security Classification Code)						
(U) Early recognition of microorganism components or products						
12. SCIENTIFIC AND TECHNOLOGICAL AREA*						
003500 Clinical medicine; 004900 Defense; 010100 Microbiology						
13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD			
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20. RESPONSIBLE DOD ORGANIZATION						
NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701						
21. RESPONSIBLE INDIVIDUAL						
NAME: Metzger, J. F. TELEPHONE: 301 663-2833						
22. GENERAL USE						
Foreign intelligence considered						
23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Puruse individual paragraphs identified by number. Puruse each with Security Classification Code.)						
(U) Counterimmunolectrophoresis; (U) BW Defense (U) Gas chromatography (U) Rickettsia (U) Bacteria (U) Antigen; (U) Antibodies						
23 (U) Develop reliable procedures for rapid identification of microbial infections, emphasizing those organisms of special importance for medical defense against BW agents.						
24 (U) Use counterimmunolectrophoresis and gas chromatography to identify infection with organisms of military significance.						
25 (U) 75 07 - 76 06 - Two animal models, one using normal and asplenic rhesus monkeys challenged intravenously with S. pneumoniae and the other using normal rhesus monkeys challenged intravenously with Salmonella typhimurium, have been developed for studying the pathogenesis of disseminated intravascular coagulation (DIC). Counterimmunolectrophoresis for detection of pneumococcal capsular polysaccharide and Limulus lysate assay for detection of Salmonella endotoxin have demonstrated antigenemia and endotoxemia in nearly all infected monkeys. However, the demonstrated antigenemia and endotoxemia occurred slightly later than bacteremia and DIC which suggests that antigenemia and endotoxemia are not primary causes of DIC as has been previously suggested.						
No further work will be carried out. The investigator is leaving the Army.						
Available to contractors upon contractee's approval.						
DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.						
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BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 03: Laboratory Identification of Biological Agents

Work Unit No. 834 03 103: Early Recognition of Microorganism Components or Products

Background:

The counterimmunoelectrophoresis (CIE) technique has been shown to be potentially useful for early, specific detection of infectious microorganisms. CIE, an immunoprecipitin technique which utilizes electric current for rapid concentration and interaction of negatively charged antigen with positively charged antibody, was selected to detect the presence of pneumococcal capsular polysaccharide (PCP) in blood samples from monkeys infected with Type I Streptococcus pneumoniae, our experimental model for investigating the pathogenesis of infection-induced disseminated intravascular coagulation (DIC). During recent years many reports of DIC associated with pneumococcal sepsis in hyposplenic individuals have appeared. These reports indicate that splenectomy predisposes patients to overwhelming pneumococcal infection. Although Rytel and coworkers¹ have implicated PCP as the initial stimulus for DIC, the pathogenesis of DIC in pneumococcal sepsis remains unclear. These authors suggest that PCP could act as the initial stimulus by itself or as part of an antigen-antibody complex. Another possibility hitherto unexamined is that whole pneumococcal organisms, and not simply PCP, are necessary to initiate DIC. In an attempt to study the pathogenesis of DIC, PCP detected with CIE, antibody titrated by passive hemagglutination (PAT), and bacteremia were measured during infections produced by challenging normal and asplenic rhesus monkeys (Macaca mulatta) with Type I S. pneumoniae.

Progress:

Ten rhesus monkeys weighing 4.5 - 6.5 kg were used; 6 were splenectomized approximately 6 mon prior to experimental use. The monkeys were allocated to 3 groups. In the first group of 4 normal monkeys, each of 3 animals was inoculated IV with 1×10^8 S. pneumoniae and 1 animal, with 1 ml physiologic saline. In the second group of 3 asplenic monkeys, 2 were injected IV with 1×10^3 S. pneumoniae and the third with 1 ml physiologic saline. The third group consisted of 3 asplenic monkeys; 2 were inoculated IV with 1×10^6 S. pneumoniae and 1 with an equal volume (1 ml) of saline. All monkeys, including controls, were treated IM with procaine penicillin G (250 mg once daily); treatment was initiated 48 to 72 hr after challenge and continued for 3-6 days. Serum PCP, PAT, bacteremia and complement components were measured twice prior to challenge and daily thereafter up to 9 days post-challenge (Table I). Samples obtained on the same bleeding schedule were

TABLE I. SUMMARY OF BACTEREMIA, ANTIGENEMIA (PCP), RECIPROCAL PROACTIVE HEMAGGLUTINATING ANTIBODY TITER (PAT) AND SERUM COMPLEMENT COMPONENTS IN NORMAL AND SPLENECTOMIZED RHESUS MONKEYS CHALLENGED IV WITH *S. PNEUMONIAE*.

Monkey Group	No.	Challenge (orgs/ml)	Maximum Temp (°C)	Maximum Bacteremia (orgs/ml)	Maximum PCP (ug/ml)	Maximum PAT	Maximum Serum Complement (mg/100 ml)		
							C3	C4	C5
Normal	1	Saline	39.6	0	0	< 10	290	40	24
	2	1×10^8	40.5	4.0×10^3	0.25	640	365	32	64
	3	1×10^8	40.5	1.1×10^6	0.5	1280	415	40	64
	4	1×10^8	40.5	1.4×10^6	0.25	640	290	36	47
Asplenic	5	Saline	39.8	0	0	< 10	290	30	13
	6	1×10^3	39.8	6.0×10^2	0	< 10	290	31	14
	7	1×10^3	39.8	0	0	20	335	24	13
Asplenic	8	Saline	39.2	0	0	< 10	340	32	12
	9	1×10^6	40.5	1.0×10^7	16	< 10	425	35	40
	10	1×10^6	40.5	2.0×10^1	0	20	515	36	29

examined for coagulation factors, kinin activation and platelet aggregation; data are also reported under Work Unit No. 834 01 112. Normal monkeys challenged with 1×10^8 *S. pneumoniae* developed bacteremia; serum levels of PCP were low and disappeared when significant PAT antibody titers for Type I PCP (prepared in our laboratory) developed. Asplenic monkeys challenged with 1×10^3 organisms developed little or no bacteremia, no antigenemia, and little or no antibody. However, an asplenic monkey (No. 9) given 1×10^6 *S. pneumoniae* developed a high level of bacteremia and extremely high serum PCP levels (maximum, 16 $\mu\text{g}/\text{ml}$) which persisted until termination of the study. Little or no antibody response was observed in asplenic monkeys.

Complement components C3, C4 and C5 were measured by radial immunodiffusion with single factor antihuman sera. The maximum serum complement levels for each monkey are shown in Table I. Complement components generally increased above baseline in the normal infected monkeys and the coagulation system activation seen in DIC were present only in the normal infected monkeys and asplenic monkeys infected with 1×10^6 *S. pneumoniae*.

To evaluate platelet aggregation as a factor in the pathogenesis of DIC, a Chrono-Log Platelet Aggregometer and Strip Chart Recorder were used. Citrated blood from 3 rhesus monkeys and 3 humans was collected in plastic syringes; platelet-rich and platelet-poor plasma were prepared by standard centrifugation techniques. The ability of platelets to aggregate when exposed to epinephrine, ADP, and collagen is shown in Table II.

TABLE II. POTENTIATION OF PLATELET AGGREGATION BY PCP.

PCP ($\mu\text{g}/\text{ml}$)	RHESUS MONKEY PLATELETS		HUMAN PLATELETS	
	ADP	Epinephrine	ADP	Epinephrine
20	-	-	+	-
40	-	-	+	-
80	-	-	+	-

A suitable collagen reaction could not be obtained despite using 2 different methods of preparation. Rhesus monkey platelets were ~5 times less sensitive to epinephrine and ADP than human platelets; this decreased response has recently been reported by Loeb and Mackey.² When PCP in a concentration of 20 $\mu\text{g}/\text{ml}$ was added prior to ADP, potentiation of platelet aggregation was consistently demonstrated with human platelets but not with those of rhesus monkeys. The potentiation of human platelet aggregation by PCP may have some etiologic significance with respect to pneumococcus-induced DIC. The failure of PCP to potentiate platelet aggregation in rhesus monkeys may be due to decreased sensitivity or a species difference in platelet behavior.

A second animal model using normal rhesus monkeys challenged with Salmonella typhimurium was also used. All studies performed with the pneumococcal model were also attempted with the Salmonella model; in addition, endotoxemia was measured by the Limulus lysate assay.

The Limulus lysate test is a sensitive technique for detecting endotoxin.³ This test is based on the observation that lysates prepared from ameboocytes of Limulus polyphemus, the horseshoe crab, gel in the presence of endotoxin. Activation of the horseshoe crab coagulation system is an in vitro phenomenon that closely resembles activation of primate coagulation in DIC.⁴ Limulus lysate (Sigma Chemical) and pyrogen-free glassware were employed to establish methods for detection of endotoxin in monkey plasma.

The technique of CIE, successfully employed for detection of PCP, was examined with antigens from S. typhimurium: an EDTA-extracted, specific lipopolysaccharide, and two commercial S. typhimurium antigens (Difco). They were tested by CIE against two commercial antisera (Difco) specific for our strain of S. typhimurium. No precipitin lines were visualized in any combination.

Seven monkeys were inoculated IV with 1×10^9 S. typhimurium and 5 with an equal volume (1 ml) of saline. The 7 experimental monkeys developed bacteremia (Table III); maximum bacterial counts did not exceed 9×10^3 /ml of blood. The PAT for Salmonella antibody was developed by Mr. Mangiafico with EDTA-extracted Salmonella antigen prepared in our laboratory. Serologic testing is in progress, but it should be noted that significant titers have been detected in sera from 2 infected monkeys thus far tested. Complement levels generally increased in infected monkeys. The Limulus lysate test demonstrated endotoxemia in 6 of 7 infected monkeys. No attempt was made to quantitate endotoxemia by serial dilution of heparinized plasma prior to assay. Evidence of DIC was manifested by activation of both the coagulation and kinin systems in all S. typhimurium-infected monkeys.

With both experimental models, diagnosis of DIC was based upon activation of kinin, coagulation and fibrinolytic systems. These changes, occurring within 24 hr after challenge, preceded detection of measurable amounts of antigen or antibody, and most closely paralleled levels of bacteremia in the blood. Complement components C4, C3 and C5 appeared to play no role in the pathogenesis of experimentally-induced DIC.

These observations have led us to conclude that: (1) PCP, endotoxin, immune complexes and complement do not appear to play primary roles in the initiation of DIC; (2) intact organisms may be the catalysts for the development of DIC; (3) the initial event in DIC appears to be Hageman-factor activation; and (4) evidence of activation of Hageman factor-dependent systems is present regardless of severity of infection.

TABLE III. SUMMARY OF BACTEREMIA, ENDOTOXEMIA, RECIPROCAL PASSIVE HEMAGGLUTINATING ANTIBODY TITER (PAT) AND SERUM COMPLEMENT COMPONENTS IN NORMAL RHESUS MONKEYS CHALLENGED IV WITH S. TYPHIMURIUM.

MONKEY GROUP	CHALLENGE orgs./ml	MAXIMUM BACTEREMIA orgs./ml	MAXIMUM PAT	MAXIMUM SERUM COMPLEMENT mg/100 ml		
				C3	C4	C5
1	Saline	0	--	32	40	20
2	1×10^9	6.2×10^2	--	320	43	30
3	1×10^9	3.3×10^3	--	430	53	32
4	Saline	0	< 10	273	39	16
5	Saline	0	< 10	320	41	24
6	1×10^9	8.6×10^3	20	490	25	40
7	1×10^9	6.0×10^1	160	330	21	58
8	Saline	0	--	300	32	16
9	Saline	0	--	240	38	28
10	1×10^9	1.0×10^3	--	300	38	68
11	1×10^9	1.57×10^3	--	342	36	48
12	1×10^9	3.4×10^2	--	330	39	57

No further work is planned; this work unit will be terminated.

Presentation:

Hawley, H. B. DIC during pneumococcal sepsis in normal and asplenic rhesus monkeys. Medical College of Wisconsin Infectious Diseases Conference, Milwaukee, WI, 13 March 1976.

Publication:

Hawley, H. B., T. Yamada, D. F. Mosher, D. P. Fine, and R. F. Berendt. 1976. Disseminated intravascular coagulopathy during experimental pneumococcal sepsis: studies in normal and asplenic rhesus monkeys. Infec. Immun., submitted.

LITERATURE CITED

1. Rytel, M. W., T. H. Dee, J. E. Ferstenfeld, and G. T. Hensley. 1974. Possible pathogenetic role of capsular antigens in fulminant pneumococcal disease with disseminated intravascular coagulation (DIC). Am. J. Med. 57:889-896.
2. Loeb, W. F., and B. Mackey. 1973. A comparative study of platelet aggregation in primates. J. Med. Primatol. 2:195-205.
3. Young, N. S., J. Levin, and R. A. Prendergast. 1972. An invertebrate coagulation system activated by endotoxin: evidence for enzymatic mediation. J. Clin. Invest. 51:1790-1797.
4. Levin, J., and F. B. Bang. 1968. Clottable protein in Limulus: its localization and kinetics of its coagulation by endotoxin. Thromb. Diath. Haemorrh. 19:186-197.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION [*] DA OD6422	2. DATE OF SUMMARY [*] 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
S. DATE PREV SURVEY 76 02 23	D. KIND OF SUMMARY D. CHANGE	S. SUMMARY SECY [*] U	S. WORK SECURITY [*] U	7. REGRADING [*] NA	8. ORIGIN INSTRN NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: [*] a. PRIMARY 61101A	PROGRAM ELEMENT PROJECT NUMBER 3A161101A91C	11. WORK AREA NUMBER 00		12. LEVEL OF SUM 4. WORK UNIT 133 834/037/103		
b. CONTRIBUTING	c. CONFIDENTIAL CARDS 114(e)(f)					
11. TITLE (Proceed with Security Classification Code) (U) Laser beam scattering for rapid identification of bacteria						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry						
13. START DATE 73 01	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY [*] DA	16. PERFORMANCE METHOD C. In-house			
17. CONTRACT/GRAANT a. DATES/EFFECTIVE: b. NUMBER: c. TYPE: d. KIND OF AWARD:		18. RESOURCES ESTIMATE EXPIRATION: FISCAL YEAR 76 CURRENT 77	19. PROFESSIONAL MAN YRS 1.0 1.0		20. FUNDS (in thousands) 40.0 40.0	
21. RESPONSIBLE DOD ORGANIZATION NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		22. PERFORMING ORGANIZATION NAME: Pathology Division USAMRIID ADDRESS: Fort Detrick, MD 21701				
23. GENERAL USE Foreign intelligence considered		PRINCIPAL INVESTIGATOR (Punish DIAW II U.S. Academic institution) NAME: Altenbernd, R. A. TELEPHONE: 301 663-5371 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: Metzger, J. F.				
		POC:DA				
24. KEYWORDS (Proceed EACH with Security Classification Code) (U) BW defense; (U) Military medicine; (U) Pathogens; (U) Identification; (U) Bacterial disease; (U) Lasers						
25. TECHNICAL OBJECTIVE [*] 26. APPROACH, 27. PROGRESS (Punish individual paragraphs identified by number. Proceed rest of each with Security Classification Code.)						
23 (U) Evaluate the usefulness of scattering of a laser beam for rapid identification of bacteria from a variety of sources. The immediate objective is to test the ability of the instrument to make error-free distinctions between 2 closely related enteric bacteria. Success would enable the scope of the program to be broadened to include many bacterial species of military medical and BW defense importance.						
24 (U) Utilizing equipment initially developed by Science Spectrum, Inc., under contract with U.S. Army Medical Research and Development Command, examine the light scattering characteristics of a variety of bacteria.						
25 (U) 75 07 - 76 06 - Technical difficulties with the laser beam apparatus have prevented any meaningful accumulation of data. It is anticipated that, in the coming year, some definitive information will be obtained.						
Available to contractors upon originator's approval.						

DD FORM 1 MAR 68 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

* U.S. GPO: 1974-840-843/8691

BODY OF REPORT

Project No. 3A061101A91C: In-House Laboratory Independent Research (U)
(3A762760A834):

Task No. 3A061101A91C 00:
(3A762760A834 03): (Laboratory Identification of Biological Agents)

Work Unit No. 91C 00 133: Laser Beam Scattering for Rapid Identification
(834 03 105): of Bacteria

Background:

Contract reports from Science Spectrum, Inc. claimed that the ratio of the third maximum to the first minimum obtained in a laser scan of a single bacterial cell was characteristic for each of the bacterial species employed. The objective of the current project was to determine if various genera of the enteric bacteria could be differentiated by this method.

Progress:

There has been no progress in the work due to frequent and extended malfunctioning of the laser beam apparatus.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION#	2. DATE OF SUMMARY#	REPORT CONTROL SYMBOL	
3. DATE PREV SURVEY	4. KIND OF SUMMARY	5. SUMMARY SCTY#	6. WORK SECURITY#	DA OF6427	76 07 01	DD-DR&E(AR)636	
76 01 30	D. CHANGE	U	U	NA	NL	D. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES: ^a	PROGRAM ELEMENT	PROJECT NUMBER		7. REGADING#	8. DODIN INSTN#	D. LEVEL OF SUM A. WORK UNIT	
6. PRIMARY	61101A	3A161101A91C		00		136	
5. CONTRIBUTING						136/03/300	
c. 114(e)	CARDS 114(e) (f)						
11. TITLE / (Proceed with Security Classification Code) (U) Host metabolism during rickettsial diseases							
12. SCIENTIFIC AND TECHNOLOGICAL AREA# 003500 Clinical medicine; 004900 Defense; 012300 Biochemistry							
13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD			
76 01	CONT	DA		C. In-house			
17. CONTRACT/GRANT							
18. DATES/EFFECTIVE:		EXPIRATION:		19. RESOURCES ESTIMATE		20. PROFESSIONAL MAN YRS	
18. NUMBER: ^b NA				FISCAL YEAR	76	0.2	5.0
19. TYPE:		6. AMOUNT:		CURRENT			
20. KIND OF AWARD:		7. CUM. AMT.		77	0.5	25.0	
21. RESPONSIBLE DOG ORGANIZATION							
NAME: ^c USA Medical Research Institute of Infectious Diseases		ADDRESS: ^c Fort Detrick, MD 21701		22. PERFORMING ORGANIZATION			
				NAME: ^c Physical Sciences Division USAMRIID			
				ADDRESS: ^c Fort Detrick, MD 21701			
23. KEYWORDS / (Proceed EACH with Security Classification Code) (U) BW defense; (U) Military medicine; (U) Pathogenesis; (U) Diagnosis; (U) Prognostic; (U) Rickettsia; (U) Vasculitis; (U) Copper							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS / (Purush individual paragraphs identified by number. Proceed each with Security Classification Code.)							
23 (U) Ascertain which alterations in host metabolism will be of diagnostic and prognostic value during rickettsial diseases, particularly those likely to be used as BW agents.							
24 (U) Measure plasma and tissue levels of trace metals and plasma levels of specific proteins, lipids and amino acids during severe rickettsial diseases in animal models.							
25 (U) 76 01 76 06 - An early increase in the plasma copper concentration, seemingly not associated with ceruloplasmin, occurs during model rickettsial spotted fever infections in guinea pigs. The increase occurs before overt illness but when there is microscopic evidence of vasculitis; thus the increase may be related to the vasculitis. Another metabolic sequela which may relate to the vasculitis is the early increase in triglycerides and free fatty acids. The connection may be that the vasculitis elicits a decrease in endothelial cell lipoprotein lipase, an enzyme considered to regulate triglyceride uptake from blood. These findings may prove to be of value in establishing optimal diagnostic and therapeutic indices.							
Publications: Proc. Soc. Exp. Biol. Med. 151:804-807, 1976. Clin. Res. 24:351A, 1976.							

BODY OF REPORT

Project No. 3A161101A91C: In-House Laboratory Independent Research (U)
(3A762760A834):

Task No. 3A161101A91C 00:
(3A762760A834 03): (Laboratory Identification of Biological Agents)

Work Unit No. 91C 00 136: Host Metabolism During Rickettsial Diseases
(834 03 300):

Background:

Extensive information has been compiled concerning metabolic alterations during bacterial illnesses in man and experimental animals which suggests that there is a stereotyped cascade of metabolic sequelae with only occasional deviations therefrom.¹ The onset and magnitude of a number of these metabolic events appear to correlate with severity of the disease process during tularemia² and many even have value as prognostic indicators during respiratory Klebsiella pneumonia³ and neonatal Escherichia coli and Hemophilus influenza⁴ infections.

In contrast little is known of the metabolic events which accompany rickettsial diseases; considering the long incubation period for rickettsial as compared to most bacterial diseases, it is quite likely that metabolic alterations might be of considerable diagnostic and prognostic value during rickettsial illnesses.

Progress:

In an initial study guinea pigs inoculated with virulent Rickettsia rickettsii responded with a significant increase in plasma Cu concentration within 1 day, preceding fever and detectable rickettsemia by 2 and 4 days, respectively. A decrease in serum Zn concentration coinciding with peak rickettsemia was detectable on day 5. Evidence of altered host nitrogen metabolism during this illness included a doubling of plasma seromucoid concentration and a significant rise in the plasma phenylalanine/tyrosine ratio.

In a subsequent study we found evidence that the rapid initial rise in plasma Cu apparently is unrelated to an increase in plasma ceruloplasmin, the major Cu-carrying protein in most animals. The fact that the increase occurs so early in the illness when there is only microscopic evidence of vasculitis tempts one to associate this increase with vasculitis. Whether the early rise in Cu is related to the vasculitis and occurs in other species remains to be determined.

In a second study we also found that plasma triglycerides and free fatty acids were elevated early in the illness. It is conceivable that the increase in plasma triglycerides is in part the result of a decrease in endothelial cell lipoprotein lipase, an enzyme considered to regulate triglyceride uptake from the blood and thus plasma triglyceride and/or free fatty acid levels may be indicators of small vessel endothelial cell dysfunction during severe rickettsial spotted fever infections.

Publications:

1. Powanda, M. C., E. C. Hauer, R. E. Whitmire, J. P. Fowler, L. A. Harris, and R. H. Kenyon. 1976. Trace metals and lipid levels during Rocky Mountain spotted fever in the guinea pig. Clin. Res. 24:351A.
2. Powanda, M. C., R. H. Kenyon, and J. B. Moe. 1976. Alterations in plasma copper, zinc, amino acids, and seromucoid during Rocky Mountain spotted fever in guinea pigs. Proc. Soc. Exp. Biol. Med. 151:804-807.

LITERATURE CITED.

1. Beisel, W. R. 1975. Metabolic response to infection. Ann. Rev. Med. 26:9-20.
2. Powanda, M. C., G. L. Cockerell, J. B. Moe, F. B. Abeles, R. S. Pekarek, and P. G. Canonico. 1975. Induced metabolic sequelae of tularemia in the rat: correlation with tissue damage. Am. J. Physiol. 229: 479-483.
3. Berendt, R. F., G. G. Long, P. G. Canonico, F. B. Abeles, and M. C. Powanda. 1976. Induced metabolic sequelae of respiratory Klebsiella pneumoniae infection in rats. Manuscript under revision.
4. Sabel, K.-G., and L. A. Hanson. 1974. The clinical usefulness of C-reactive protein (CRP) determinations in bacterial meningitis and septicemia in infancy. Acta. Paediatr. Scand. 63:381-388.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ⁹ DA 0C6418	2. DATE OF SUMMARY ⁹ 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SURVEY 75 07 01	4. KIND OF SUMMARY H. TERMINATION	5. SUMMARY SEC ¹⁰ U	6. WORK SECURITY ¹⁰ U	7. REGADING ⁹ NA	8. DA DISP'N INSTN ⁹ NL	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: 62760A	PROGRAM ELEMENT	PROJECT NUMBER 3A762760A834		TASK AREA NUMBER 03	11. LEVEL OF SUM A. WORK UNIT 403	
12. TITLE (Pencile with Security Classification Code) ⁹ (U) Separation and purification of arbovirus agents and their rapid detection in clinical specimens						
13. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁹ 003500 Clinical medicine; 004900 Defense; 010100 Microbiology						
14. START DATE 61 11	15. ESTIMATED COMPLETION DATE CONT		16. FUNDING AGENCY DA	17. PERFORMANCE METHOD C. In-house		
18. CONTRACT/GRANT			19. RESOURCES ESTIMATE	& PROFESSIONAL MAN YRS		B. FUNDS (in thousands)
20. DATES/EFFECTIVE:			PREVIOUS	1.0		123.6
21. NUMBER: NA			FISCAL YEAR	CURRENCY		
22. TYPE:			76			
23. AMOUNT:			77	0		0
24. KIND OF AWARD:			F. CUM. AMT.	25. PERFORMING ORGANIZATION		
26. RESPONSIBLE DOB ORGANIZATION			NAME: Virology Division USA Medical Research Institute of Infectious Diseases ADDRESS: USAMRIID Fort Detrick, MD 21701			
27. RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833			NAME: Levitt, N. H. TELEPHONE: 301 663-7241 SOCIAL SECURITY ACCOUNT NUMBER:			
28. GENERAL USE Foreign intelligence considered			ASSOCIATE INVESTIGATORS NAME:			
POC:DA						
29. DECLASSIFICATION (Pencile EACH item with Security Classification Code) (U) Cellulose acetate electrophoresis; (U) BW Defense; (U) Arboviruses; (U) Serology; (U) Antigen-antibody reactions; (U) Fluorescent antibody; (U) Encephalitis, equine (WEE)						
30. TECHNICAL OBJECTIVE, 26 APPROACH, 28 PROGRESS (Pencile individual paragraphs identified by number. Pencile last of each with Security Classification Code.) 23 (U) Develop methodology for the rapid detection and identification of group A arboviruses causing militarily significant disease, especially those believed to be important for medical defense against BW agents.						
24 (U) Viruses propagated in tissue culture are identified by the detection of specific antibody complexed to radioactive 125-I labeled gamma globulin.						
25 (U) 75 07 - 76 06 - A solid-phase radioimmunoassay procedure was developed which can detect and identify WEE virus in clinical specimens within 24 hours. The bulk of this work was reported in last year's annual report. A manuscript describing the procedure has been submitted for publication to The Journal of Clinical Microbiology. No work has been performed on this work unit due to the reassignment of the principal investigator to another project. No further work will be performed in the foreseeable future. This work unit will be terminated.						
Publication: J. Clin. Microbiol. 4: in press.						
Available to contractors upon contractor's approval.						
DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE. * U.S. GPO: 1974-840-843/6691						

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BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 03: Laboratory Identification of Biological Agents

Work Unit No. 834 03 403: Separation and Purification of Arbovirus Agents
and their Rapid Detection in Clinical Specimens

Background and Progress:

A solid-phase radioimmunoassay procedure was developed which can detect and identify Western equine encephalitis virus in clinical specimens within 24 hours. The bulk of this work was reported in last year's annual report. A manuscript describing the procedure has been submitted for publication to The Journal of Clinical Microbiology. No work has been performed on this work unit due to the reassignment of the principal investigator to another project. No further work will be performed in the foreseeable future. This work unit will be terminated.

Publication:

Levitt, N. H., H. V. Miller, and G. A. Eddy. 1976. Solid-phase radioimmunoassay for rapid detection and identification of Western equine encephalomyelitis virus. J. Clin. Microbiol. 4: in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
3. DATE PREV SUBMT	4. KIND OF SUMMARY	5. SUMMARY ACTV ^b	6. WORK SECURITY ^c	DA OC6420	76 07 01	DD-DR&E(AR)636
75 07 01	D. CHANGE	U	U	NA	NL	8. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
9. NO./CODES ^d	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	9. LEVEL OF SUM A. WORK UNIT	
D. PRIMARY	62 760A	3A762 760A834		03	405	
D. CONTRIBUTING						
C. APPROV ^e	CARDS 114(e)(f)					
11. TITLE (Provide with Security Classification Code) (U) Experimental Bolivian hemorrhagic fever: pathogenesis and vaccine development						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS 003500 Clinical medicine; 004900 Defense; 010100 Microbiology						
13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD			
71 06	CONT	DA	C. In-house			
17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS		20. FUNDS (in thousands)
B. DATES/EFFECTIVE:		FISCAL	PRECEDING	3.0	306.9	
D. NUMBER: ^f NA		YEAR	CURRENT	2.0	104.0	
C. TYPE:		E. AMOUNT:				
G. KIND OF AWARD:		F. CUM. AMT.				
21. RESPONSIBLE DOD ORGANIZATION		22. PERFORMING ORGANIZATION		23. PRINCIPAL INVESTIGATOR (Provide name if U.S. Government institution)		
NAME: ^g USA Medical Research Institute of Infectious Diseases ADDRESS: ^g Fort Detrick, MD 21701		NAME: ^g Virology Division USAMRIID ADDRESS: ^g Fort Detrick, MD 21701		NAME: ^g Eddy, G. A. TELEPHONE: 301 663-7241		
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833				SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: Scott, S. K. NAME: Wagner, F. M.		
24. GENERAL USE Foreign intelligence considered				POC:DA		
25. KEYWORD (Provide each with Security Classification Code) (U) BW defense; (U) Military medicine; (U) Bolivian hemorrhagic fever (BHF); (U) Machupo virus; (U) Arenavirus; (U) Viral vaccines						
26. TECHNICAL OBJECTIVE, ^h 27. APPROACH, 28. PROGRESS (Provide individual paragraphs identified by number. Provide test of each with Security Classification Code.)						
23 (U) Define mechanism of pathogenesis and develop a vaccine against BHF. Establish a model system for development of inactivated vaccine against related diseases including Argentine hemorrhagic fever and Lassa fever.						
24 (U) Using available models for BHF test live attenuated and inactivated vaccines and study pathogenic mechanisms. Study virus replication; test a variety of virus seeds and clones in the available cell cultures to select an optimal combination for producing sufficient antigen to use as an inactivated vaccine. Develop alternative models for BHF.						
25 (U) 75 07 - 76 06 - The African green monkey (<i>Cercopithecus</i>) is a useful model for studying BHF; and a fatal hemorrhagic guinea pig model was developed. Data strongly suggest that disseminated intravascular coagulation, or certain aspects of it, are involved in deaths of rhesus monkeys with experimental BHF. A reproducible late encephalitis has been described which is dependent upon the administration of specific antibody in high doses for induction in infected monkeys. Both live, attenuated and inactivated experimental vaccines have been used to protect monkeys against challenge with the etiologic virus of BHF, Machupo virus. The attenuated virus appears to be mediated through defective interfering virus and the inactivated vaccines does not appear to depend upon the development of antibody in the recipient monkeys for efficacy.						
Publications: J. Infect. Dis. 133:57-62, 1976. Am. J. Pathol. 84:211-224, 1976. Bull. WHO. 52:517-521, 723-727, 1975; Am. J. Trop. Med. Hyg. 25: in press, 1976.						
29. APPROVAL & SIGNATURES DD FORM 1 MAR 68 1498						
PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.						
* U.S. GPO: 1974-840-843/8691						

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 03: Laboratory Identification of Biological Agents

Work Unit No. 834 03 405: Experimental Bolivian Hemorrhagic Fever: Pathogenesis and Vaccine Development

Background:

Because of the clinical and histopathological evidence of coagulopathy in Machupo virus-infected rhesus monkeys, studies were designed to elucidate the mechanism of the coagulation disorder. Specifically, the studies were directed toward determining whether the phenomenon of disseminated intravascular coagulation (DIC) has a role in the pathogenesis of this disease in the monkey.

It has been reported in the literature that hypotension accompanied by defervescence was experienced by about half of the human cases of BHF in Bolivia. This hypotension was reported to vary from mild and transitory to severe and progressive, in the latter case culminating in death from severe hypovolemic shock. Because of these reports, it was determined that any study of the coagulation mechanism in Machupo virus-inoculated rhesus monkeys should be preceded by studies directed toward determining whether the monkeys also develop hypotension during the course of the disease, and if so, to what degree and at what time.

Other studies in pathogenesis were directed toward the phenomenon of late encephalitis, attempting to induce the phenomenon in monkeys by inoculating high and low doses of specific antibody into Machupo virus-infected monkeys.

Vaccine development efforts continued on both a live attenuated virus and an experimental inactivated product. Our principal objective is to develop a methodology for preparing vaccines against the virulent arenaviruses.

Progress:

Blood Pressure Studies. Blood pressures were recorded daily at 0800 hours on a total of 9 rhesus monkeys, 3 saline controls and 6 Machupo virus inoculated beginning on day -4 of the studies. Each monkey had surgically implanted venous and arterial catheters. Clinical signs were recorded daily. Viremia and serum neutralizing antibody assays and complete blood counts were also done.

The virus-inoculated monkeys developed typical clinical signs of the disease. The course of disease, however, was more acute than might be expected in caged monkeys without surgically-implanted IV catheters. The mean time to death for the group was 13 days; 4 out of 6 were viremic on day 5 postchallenge. Blood pressure measurements for the 2 groups of monkeys are shown in Table I. The

systolic and diastolic pressures were taken directly from the blood pressure recordings, and the mean blood pressures were calculated. There was a variation in mean blood pressure from baseline (days -4 and -0) in both groups when compared to the first 7 or 8 days postchallenge. Because of this variation, a 1-way analysis of variance was applied to the data, comparing the group mean differences in mean blood pressure on each given postinoculation day from each group's own mean baseline values, to determine significant differences. Mean blood pressures for group II monkeys (virus-inoculated) began a gradual decline on day 7 but a 99% confidence level difference was not attained until day 10. Most significantly, the onset of this gradually-developing hypotensive state coincided with the onset of defervescence in the monkeys at day 7. No severe hypotension (which might be defined as the shock level, or roughly a mean blood pressure of ≤ 50 mm Hg in an individual monkey) was experienced until the terminal stage of the illness in each virus-infected monkey. This would imply that there should be no difficulties in interpreting data obtained from blood coagulation studies in Machupo virus-infected monkeys, at least not until the last 1 or 2 days of illness.

TABLE 1. MEAN BLOOD PRESSURES^a IN CHAIRED CONTROL (n=3) VS. MACHUPO VIRUS INFECTED (n=6) RHESUS MONKEYS

DAY OF ^b PROJECT	MEAN BLOOD PRESSURE		P	DEAD GROUP II/TOTAL
	Group I	Group II		
-4	119	110		0/6
-3	119	114		
-2	114	114		
-1	117	113		
0	104	108		
1	101	101		
2	99	98		
3	108	94		
4	106	101		
5	106	99		
6	104	98		
7	112	91	<0.1	
8	106	90		1/6
9	117	80	<0.1	
10	117	76	<0.01	
11	110	76	<0.05	
12	109	64	<0.05	2/6
13	114	65	<0.05	3/6
14	113	60	<0.01	5/6
15				
16				6/6

^a Mean pressure = diastolic + pulse pressure (pulse pressure = systolic - diastolic)

^b Virus inoculated on day 0

From these data, however, and from the clinical condition of the monkeys, severely decreased hematocrit values, loss of weight, dehydration, poor tissue perfusion, paleness, etc., it appears that monkeys are dying in a state of hypovolemic shock. Circulating blood volume studies would lend further proof to this assumption. The majority of human patients with DIC who die seem to do so from irreversible hypovolemic shock, not from massive hemorrhaging. Thus it is likely that the Machupo virus-infected rhesus monkey may be experiencing episodes of DIC.

Coagulation Studies. To better understand the pathogenesis of Machupo virus infection in rhesus monkeys, a basic study was designed to evaluate the coagulation mechanism. A total of 8 virus-infected and 4 control monkeys were used, with the control monkeys from the previous blood pressure studies being re-used (as virus-infected monkeys). Two experiments were to be conducted, with 2 control and 4 infected monkeys per run. The second run is still in progress and the data, are incomplete at this time. Clinical indices studied included platelet counts, hematocrits, serum electrolytes (Na^+ , K^+), sorbitol dehydrogenase (SDH) enzyme, FSP, fibrinogen levels, prothrombin times (PT), automated partial thromboplastin times (APTT), viremia, and serum neutralizing antibody. The monkeys were bled on days -4, -2, and 0 of the study to establish baseline values. Inoculations of either 10^3 PFU of Machupo virus or normal saline were given SC after the day 0 bleeding.

It was hypothesized that DIC has a role in the pathogenesis of BHF in rhesus monkeys. The studies described above were designed specifically to test this hypothesis. There is no one single laboratory test for DIC, but its diagnosis can be made by the use of screening tests such as PT, APTT, platelet counts, and fibrinogen levels. The PT measures blood coagulation factors, I, II, V, VII, X (extrinsic pathway) and is also sensitive to FSP. The APTT is sensitive to deficiencies of factors V, VIII, IX, X, XI, and XII (intrinsic pathway). The combination of a prolonged PT (or APTT), thrombocytopenia, and hypofibrinogenemia (all with changes beyond the range of 1 SD from baseline) would confirm a diagnosis of DIC. If any one of these is normal, an additional confirmatory test such as FSP is needed.

The data accumulated from the first 6 monkeys is incomplete, as the fibrinogen assays and FSP tests have not yet been completed. However, certain inferences can be made from the data accumulated thus far. Only 1 of the 4 virus-infected monkeys developed a prolonged PT. However, all 4 developed prolonged APTT values beginning on day 7 postchallenge (Table II), which indicates a defect in the intrinsic pathway of the coagulation mechanism. Several other clinical indices changed at about the same time as the APTT values. At day 7, platelet counts and hematocrits began to decrease. Terminally, all 4 monkeys were thrombocytopenic and anemic. At day 5 - 7, serum SDH values in the 4 infected monkeys began to increase sharply (Table III), indicating an increasingly severe hepatic necrosis and/or hepatitis (SDH being an essentially liver specific enzyme). Serum albumin concentrations (Table III) decreased on day 7 postchallenge, possibly indicating compromised liver function and correlating well with the SDH results. There were probably no significant changes in serum electrolyte (Na^+ , K^+) concentrations, although there does seem to be a slight decrease in Na^+ values terminally. Also, between days 7 and 9, the 4 monkeys were in their peak fever period (values not shown).

TABLE II. COAGULATION TESTS (PT AND APTT) IN 2 CONTROL AND 4 MACHUPO VIRUS INFECTED RHESUS MONKEYS

DAY OF STUDY	MEAN PT + SE		HUMAN CONTROL PLASMA	MEAN APTT + SE		HUMAN CONTROL PLASMA	SURVIVORS GROUP II
	Control	Infected seconds		Control	Infected seconds		
-4	11.4	11.2 + 0.1	11.4	28.9	28.3 + 1	28.8	4
-2	10.9	10.4 + 0.1	10.4	28.8	28.0 + 1	28.6	4
0	11.9	11.0 + 0.1	11.4	29.7	27.7 + 1	29.0	4
3	11.5	10.9 + 0.1	11.0	29.4	28.3 + 2	28.5	4
5	10.4	10.6 + 0.2	12.4	27.1	30.4 + 2	24.9	4
7	10.7	11.3 + 0.4	12.4	26.4	39.2 + 2	22.9	4
10	10.6	10.6 + 0.4	10.9	26.9	65.7 + 16	25.9	4
12	10.0	12.0 + 0.2	11.0	27.9	62.5 + 10	24.9	4
14	10.2	10.4 + 0.2	11.5	25.7	50.1 + 3	25.9	3
17	9.9	11.3 + 0.1	11.4	27.5	50.4 + 3	26.4	3

TABLE III. SERUM SORBITAL DEHYDROGENASE AND ALBUMIN CONCENTRATIONS IN 2 CONTROL AND 4 MACHUPO VIRUS INFECTED RHESUS MONKEYS

DAY OF STUDY	SDH (Sigma U + SE)		ALBUMIN (g/dl + SE)	
	Control	Infected	Group I	Group II
-4	75	75.4 + 14	3.9	3.9 + 0.2
-2	139.6	115.5 + 9	3.8	3.9 + 0.1
0	137.4	72.7 + 12	3.8	4.3 + 0.5
3	69.6	155.7 + 75	4.2	3.0 + 0.1
5	121.0	206.6 + 48	3.3	2.8 + 0.2
7	69.6	238.8 + 74	3.0	2.3 + 0.1
10	145.3	239.2 + 43	3.3	2.5 + 0.2
12	69.6	514.0 + 72	3.3	2.1 + 0.2
14	34.9	508.8 + 57	3.6	2.1 + 0.2
17	74.8	1,579.5 + 396	3.8	2.0 + 0.1

From the blood pressure studies previously described, it was shown that the infected monkeys did not experience severe hypotension until the terminal 1 or 2 days of the illness. Thus, the changes seen in the coagulation studies could be ascribed to viral effects on organs and tissues in the monkeys.

and not merely to severe hypotension. The monkeys apparently have a defect in the intrinsic limb of the coagulation mechanism, and at the same time show evidence of liver damage. The initiating mechanism for the defect in the intrinsic system may be due to endothelial cell injury which would activate factor XII (Hageman factor) and the intrinsic clotting system, or to platelet or red cell injury, which would have the same effect. The fact that the monkeys show evidence of liver damage is difficult to interpret in view of the coagulation studies, because ordinarily they would be accompanied by prolonged PT values. Only 1 of 4 monkeys showed a prolonged PT (the last day of illness). However, the studies have determined that there is a coagulation defect (in the intrinsic system) and when the fibrinogen and FSP tests are completed, a diagnosis of DIC may be possible.

Late Encephalitis. We have attempted to develop a reliable model for late encephalitis which would allow us to produce the phenomenon of Machupo virus-induced late encephalitis in monkeys and other animals. Data from last year's report suggested that monkeys inoculated with virus and immune globulin at approximately the same time would sometimes exhibit no acute illness, but they might develop late encephalitis as late as 2 to 3 months.

The data in Table IV show the composite results of 2 separate studies. All monkeys were inoculated with Machupo virus and received the indicated dose of immune globulin 4 hr later.

TABLE IV. OCCURRENCE OF DISEASE IN PASSIVELY PROTECTED MACHUPO VIRUS-INOCULATED MONKEYS

IMMUNE GLOBULIN DOSE ^a ml/kg	ACUTE DISEASE/ TOTAL (DEATHS)	MEAN DAY OF DEATH	NEUROLOGIC DISEASE/ TOTAL (DEATHS)	MEAN DAY OF DEATH
1.5	0/8 (2)		7/8 (5)	50
0.5	0/3 (0)		1/3 (1)	42
0.15	4/8 (2)	26	0/6 (0)	

^a Monkeys inoculated with 1000 PFU Machupo virus 4 hr later they were given indicated dose of immune globulin.

The monkeys receiving the 2 higher doses of immune globulin were protected against the acute disease. Four of the 8 monkeys receiving the lowest dosage of globulin became acutely ill and 2 died. There was a clear direct relationship between dose of immune globulin and protection against acute illness. The immune globulin did not protect against late encephalitis. On the contrary the monkeys that received the highest dosage of immune globulin showed a significantly higher incidence (7 of 8 monkeys) of late encephalitis than did the 6 monkeys which survived the acute disease (none with late encephalitis). We have no explanation for this unanticipation phenomenon.

TABLE V. PRELIMINARY STUDIES ON CHICK CELL PASSAGED MACHUPO VIRUS IN RHESUS MONKEYS

PASSAGE LEVEL	YIELD IN CHICK CELLS \log_{10} PFU	ILLNESS IN MONKEYS	DAYS VIREMIC/ DAYS TESTED	SURVIVORS/ TOTAL
0	1-2	Severe	9/9	0/2
18	3-4	Severe	8/9	1/2
23	4-5	Moderate	6/10	2/2
31	4-5	Mild	2/10	2/2

Vaccine Development Against BHF. Two types of vaccine have been explored as immunogens against BHF. A virus strain has been successively passaged in chick embryo cell culture to attempt to attenuate it. Upon passage the virus was progressively attenuated through passage 31 (Table V),¹ monkey virulence and viremia progressively decreased with passage. At passage 31 the virus was still not sufficiently attenuated. However, with further passage we observed a partial return to the virulent state. Moreover, it was found that the number of defective virions in the passaged virus was almost $9 \log_{10}$ in titer and that the number of defective particles corresponded roughly to the level of attenuation. Although the virus is now at passage level 60, we are discouraged about the possibility of live attenuated vaccines for arenavirus disease. The genetic instability, and the facility of arenaviruses to adapt to new host systems indicates that any live attenuated virus will require decades to be developed.

TABLE VI. EFFICACY OF KILLED BHF VACCINE IN MONKEYS CHALLENGED ON DAY 0

VACCINE DOSE ml	N	NO. WITH N AB ^a BY DAYS			NO. WITH SEVERE ACUTE ILLNESS (Deaths)		LATE SEQUELAE (Deaths)
		0	7	10	0	(0)	
3.0	4	1	4	4	0	(0)	0
0.3	4	0	0	3	1	(0)	1 (1)

^a Monkeys were challenged on day 0, 3 weeks after vaccination and they developed antibody by the indicated day.

As an alternative we have attempted to make experimental killed vaccines. Results to date have been most encouraging in monkeys. We inoculated rhesus monkeys with a single dose of either 3 ml or 0.3 ml of vaccine (Table VI); all were challenged 3 weeks later. Only 1 of 8 monkeys became ill despite the absence of detectable neutralizing antibody in most of them at the time of challenge. This suggests that priming of immune cells by the killed vaccine

was adequate to provoke an early antibody response to the challenge virus, and that this secondary type of immune response protected 7 of 8 monkeys against severe disease. CPT Elwell reported that the surviving monkeys exhibited no histological evidence of encephalitis or vasculitis.

Alternative Model System. In addition to the rhesus and cynomolgus monkeys which we have used almost exclusively to study BHF, we have recently developed 2 new model systems. We have described BHF in terms of clinical signs, hematologic changes, viremia and antibody response in the African green (*Cercopithecus*) monkey. In general, we observed a similar series of clinical and virologic events in this monkey as we had reported previously in macaques. The disease course was briefer and more severe, but it was entirely consistent with the human illness.

Although we had not previously found guinea pigs to be a satisfactory model for BHF, we recently began to passage the virus in guinea pigs, using spleen material from sick animals. Within 3 passages the virus became highly virulent for guinea pigs. By the 5th passage we found that the virus was uniformly lethal for all guinea pigs. The availability of a lethal guinea pig model for hemorrhagic disease is very useful for pathogenesis and virulence experiments and particularly for measuring vaccine efficacy. It will permit us to do many experiments that are impractical to perform in monkeys.

Publications:

1. Kastello, M. D., G. A. Eddy, and R. W. Kuehne. 1976. A rhesus monkey model for the study of Bolivian hemorrhagic fever. *J. Infect. Dis.* 133: 57-62.
2. McLeod, Jr., C. G., J. L. Stookey, G. A. Eddy, and S. K. Scott. 1976. Pathology of chronic Bolivian hemorrhagic fever in the rhesus monkey. *Am. J. Pathol.* 84:211-224.
3. Eddy, G. A., S. K. Scott, F. S. Wagner, and O. M. Brand. 1976. Pathogenesis of Machupo virus infection in primates. *Bull. WHO (Suppl.)*, in press.
4. Eddy, G. A., F. S. Wagner, S. K. Scott, and B. J. Mahlandt. 1976. Protection of monkeys against Machupo virus by the passive administration of Bolivian hemorrhagic fever immune globulin. *Bull. WHO (Suppl.)*, in press.
5. Scott, S. K., G. A. Eddy, and O. M. Brand. 1976. The African green monkey as a model for Bolivian hemorrhagic fever. *Am. J. Trop. Med. Hyg.* 25: in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ² DA OF6413	2. DATE OF SUMMARY ³ 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
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C. TYPE: F. AMOUNT: E. CUM. AMT.		NAME ¹⁰ Metzger, J. F.		ADDRESS ¹⁰ USAMRIID		
G. KIND OF AWARD: F. CUM. AMT.		NAME ¹⁰ 301 663-2833		ADDRESS ¹⁰ Fort Detrick, MD 21701		
21. GENERAL USE Foreign intelligence considered		NAME ¹⁰ Veltri, B. J.		PRINCIPAL INVESTIGATOR (PUNISH NAME IF U.S. Academic Institution) SOCIAL SECURITY ACCOUNT NUMBER:		
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BODY OF REPORT

Project No. 3A061101A91C: In-House Laboratory Independent Research (U)
(3A762760A834):

Task No. 3A061101A91C 00:
(3A762760A834 03): (Laboratory Identification of Biological Agents)

Work Unit No. 91C 00 131: Morphogenesis and Morphology of Venezuelan
(834 03 407): Equine Encephalomyelitis Virus

Background:

VEE virus, a member of the Togavirus group, subgroup A, propagates in several primary and continuous cell cultures. The structure and morphogenesis may be similar to other viruses in the same subgroup. Togaviruses are enveloped viruses which contain 2 glycoproteins and a nucleocapsid protein.¹ The nucleocapsid possesses a single internal protein which is associated with the RNA, and is hydrophilic. Previous studies with this group of viruses demonstrated that the envelope is acquired as the nucleocapsid buds through cell membrane, cytoplasmic vacuole membrane and/or perhaps endoplasmic reticulum. There is evidence that glycoprotein spikes do not penetrate deeply into the lipid layer.²

Progress:

Viral elementary bodies are formed from a cylindrical type structure from within cell vacuoles and within the cell cytoplasm probably close to polyribosomal accumulation.³ Previously formed viral glycoproteins migrate to designated areas of the cell membrane.^{2,4} Nucleocapsids attach to the membrane protein and the viral glycoprotein develops into visible projections (peplomers) on the outside of the plasma membrane. Budding occurs as sort of a "reverse cytosis" in which there is evagination of the altered area of the membrane and the virion, enclosing its nucleocapsid.²

Freeze-etching of fractured VEE virus-infected cells reveals new information about Togavirus, subgroup A, morphology.⁵ Utilizing this technique we find the fractured nucleocapsid to measure ~ 55-60 nm in diameter. Nucleocapsids are composed of subunits measuring ~ 8 nm in diameter. These subunits are organized in a symmetrical array suggestive of an icosahedral design. Peplomers are observed attached to a lipid layer which appears to be loosely attached to the capsomeres. The nucleocapsid appears to adhere to the inner surface of the cell membrane before virus maturation. We assume this to be so because there is always a fracture plane between the capsomeres and peplomers. There is probably no protein in the lipid layer. The lipid layer seems to be attached to the peplomers, but not to the nucleocapsid.

It is difficult to fracture pure viruses unless one uses high concentrations of glycerol. To obtain double replicas of fractured pure virus requires time and patience. Fracture planes revealing the outer and inner surface of VEE virus are common. We have observed fractures separating capsomers and peplomers, but could not obtain a fracture through the viral membrane. The nearest fracture through the virus core reveals a round dense area close to the center of the virus. It measures ~ 20-22 nm in diameter and appears to be protruding upward when viewed stereoscopically. We believe this is probably a tightly packed protein-nucleic acid complex. In core fractures we also observed strands measuring ~ 1-2 nm in diameter and 20-25 nm in length.

The anatomic detail of infected cell membrane and virus morphology suggest a rather different interpretation and analysis using freeze-etch technique compared to other methods. Fractures always occur in the weakest area of the host cell and virus, therefore revealing the same fracture plane. Suggested technical refinements could well include use of ion milling to reveal surfaces not yet exposed by freeze-fracture. A manuscript entitled, "Morphology and morphogenesis of Venezuelan equine encephalomyelitis virus as revealed by stereography of shadowed replicas from complementary freeze-etched surfaces," is being written.

Publications:

None

LITERATURE CITED

1. Schlesinger, M. J., S. Schlesinger, and B. W. Burge. 1972. Identification of a second glycoprotein in Sindbis virus. *Virology* 47: 539-541.
2. Birdwell, C. R., and J. H. Strauss. 1974. Replication of Sindbis virus. IV. Electron microscopy study of the insertion of viral glycoproteins into the surface of infected chick cells. *J. Virol.* 14:366-374.
3. Bykovsky, A. F., F. I. Yershov, and V. M. Zhdanov. 1969. Morphogenesis of Venezuelan equine encephalomyelitis virus. *J. Virol.* 4:496-504.
4. Brown, D. T., M. R. F. Waite, and E. R. Pfefferkorn. 1972. Morphology and morphogenesis of Sindbis virus as seen with freeze-etching techniques. *J. Virol.* 10:524-536.
5. Veltri, B. J., and J. H. McAlear. 1972. Wall and plasma membrane structures of Hydrogenomonas eutropha as revealed by stereography of replicas from complementary freeze-etched surfaces. *J. Gen. Microbiol.* 70:31-41.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ⁹ DA OG6411	2. DATE OF SUMMARY ⁹ 76 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SURVEY 76 02 18	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCRT ⁹ U	6. WORK SECURITY ⁹ U	7. REGRADING ⁹ NA	8. DISSEM INSTN ⁹ NL	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES ⁹ a. PRIMARY 61101A	PROGRAM ELEMENT PROJECT NUMBER 3A161101A91C			TASK AREA NUMBER 00	WORK UNIT NUMBER 139	
b. CONTRIBUTING					834/03/408	
c. CONFIDENTIAL CARDS 114(e)(f)						
11. TITLE (Provide with Security Classification Code) (U) Rapid diagnostic method based on singlet oxygen fluorescence						
12. SCIENTIFIC AND TECHNOLOGICAL AREA ⁹ 003500 Clinical medicine; 004900 Defense; 010100 Microbiology						
13. START DATE 76 02	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-house			
17. CONTRACT/GRAANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS		
b. DATES/EFFECTIVE: b. NUMBER: c. TYPE: d. KIND OF AWARD:		EXPIRATION: NA	FISCAL YEAR	PREVIOUS 76	0.1	5.0
		AMOUNT: f. CUM. AMT.	CURRENT	77	0.1	5.0
20. RESPONSIBLE DOG ORGANIZATION		21. PERFORMING ORGANIZATION				
NAME: USA Medical Research Institute of Infectious Diseases ADDRESS: Fort Detrick, MD 21701		NAME: Bacteriology Division USAMRIID ADDRESS: Fort Detrick, MD 21701				
RESPONSIBLE INDIVIDUAL NAME: Metzger, J. F. TELEPHONE: 301 663-2833		PRINCIPAL INVESTIGATOR (Provide name if U.S. Academic institution) NAME: Canonicco, P. G. TELEPHONE: 301 663-7341 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: Powanda, M. C. NAME: Levitt, N. H.				
22. GENERAL USE Foreign intelligence considered		POC:DA				
23. KEYWORD (Provide each with Security Classification Code) (U) BW defense; (U) Military medicine; (U) Diagnosis; (U) Singlet oxygen; (U) Fluorescence; (U) Phagocytosis; (U) Virus						
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Provide individual paragraphs identified by number. Provide last of each with Security Classification Code.) 23. (U) Develop a rapid diagnostic procedure for detection of viruses or virus-specific antibody. This will aid in the early diagnosis of infections caused by potential BW agents.						
24 (U) Singlet oxygen fluorescence occurring in polymorphonuclear leukocytes during in vitro phagocytosis of antibody-virus complexes is used to assay either antibody or virus.						
25 (U) 76 02 - 76 06 - Monkey peripheral leukocytes were added to a scintillation vial along with diluted sera or viral, rickettsial or bacterial particles, Luminol-saturated fetal calf serum and Hanks' balanced salt solution. Chemiluminescence (CL) was monitored. When opsonized Francisella tularensis, Rickettsia rickettsii or Venezuelan equine encephalomyelitis or Pichinde virus was used, CL titers were much higher with immune serum than nonimmune.						
It has been shown that phagocytosis of opsonized viruses by polymorphonuclear leukocytes may be detected by CL; titers are similar to, or higher than by complement fixation.						
Comments to supervisor upon completion of survey.						

DD FORM 1 MAR 68 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

* U.S. GPO: 1974-840-843/8691

BODY OF REPORT

**Project No. 3A061101A91C: In-House Laboratory Independent Research (U)
(3A762760A834):**

**Task No. 3A061101A91C 00:
(3A762760A834 03): (Laboratory Identification of Biological Agents)**

**Work Unit No. 91C 00 139: Rapid Diagnostic Method Based on Singlet Oxygen
(834 03 408): Fluorescence**

Background:

Current serological methods for detection of virus specific antibody (Ab) are often either characterized by slow, time-consuming procedures, or are inadequately specific and unrelated to the immune status of the host. Recently, a radiometabolic assay was developed for detection of bacteria specific antibody.¹ This assay consists of incubating a known quantity of bacteria with specific antibody to form antigen-antibody complexes (i.e., opsonized bacteria). Addition of polymorphonuclear phagocytes (PMN) and radiolabeled glucose (G-1-¹⁴C) to the incubation medium results in phagocytosis of the opsonized bacteria. Energy generated by oxidation of G-1-¹⁴C to ¹⁴CO₂ via the hexose monophosphate (HMP) shunt of the PMN is required for phagocytosis of the opsonized bacteria to occur. Since the amount of energy required is proportional to the number and size of ingested complexes, the amount of G-1-¹⁴C oxidized to ¹⁴CO₂ becomes a measure of the quantity of available opsonizing Ab.

The detection of viral specific Ab by this procedure, however, has proved impractical since the amplification system, i.e., the amount of G-1-¹⁴C oxidized to ¹⁴CO₂ required to ingest opsonized viruses, is insignificant compared to that required for ingestion of the much larger bacteria-Ab complexes. Recently, however, phagocytosis of Ag-Ab complexes has been shown to elicit chemiluminescence from PMN,² which reflects the generation of singlet oxygen, correlates with HMP shunt activity and should provide a more sensitive amplification system than ¹⁴CO₂ measurement. This finding opens the possibility that ingestion of opsonized viruses by PMN may be detected by this more sensitive procedure.

Progress:

PMN (3-5 x 10⁶) isolated from monkey peripheral blood were added to a 15-ml liquid scintillation vial along with 0.05 ml of appropriately diluted test sera or viral, rickettsial or bacterial particles, 1 ml of Luminol-saturated fetal calf serum and Hanks' balanced salt solution to a volume of 5 ml. Chemiluminescence (CL) was monitored using a Packard scintillation spectrometer, model 3320, operated in the in-coincidence summation mode at room temperature.

When opsonized Francisella tularensis organisms (3×10^8) were used, CL production was $> 10^7$ CPM within 25 min, in contrast to 10^4 CPM when nonimmune serum was used. CL production during the first 100 min of incubation with Rickettsia rickettsii organisms was 5 X greater in the presence of immune serum than control serum. Opsonized (8×10^9) VEE virus resulted in a CL peak $> 140,000$ CPM after 240 min of incubation compared to 3000 CPM for nonimmune serum. Pichinde virus immune serum had a CL production peak of $\sim 10,000$ CPM at an equivalent time period. Titration of the Pichinde anti-serum gave CL titers similar to, or of greater magnitude than, those obtained by CF serology.

These findings demonstrate that phagocytosis of opsonized viruses by PMN may be detected by measurements of CL, and thus make possible the development of a new assay for the rapid detection of specific viral antibody. The technique appears to be as sensitive as other serological methods in current use.

Publications:

None.

LITERATURE CITED

1. Canonico, P. G., A. T. McManus, J. A. Mangiafico, L. S. Sammons, V. G. McGann, and H. G. Dangerfield. 1975. Temporal appearance of opsonizing antibody to Francisella tularensis: detection by a radiometabolic assay. Infect. Immun. 11:466-469.
2. Allen, R. C., R. L. Stjernholm, and R. H. Steele. 1972. Evidence for the generation of an electronic excitation state(s) in human polymorpho-nuclear leukocytes and its participation in bactericidal activity. Biochem. Biophys. Res. Commun. 47:679-684.

APPENDIX A

TSP-05

TECHNICAL SUPPORT FOR THE BIOASSAY OF INTERFERONS

Bruno J. Luscri, Ph.D.
Virology DivisionObjectives:

To determine the sensitivity of selected arenaviruses to an interferon of human origin; and to examine the ability of arenaviruses to induce interferon production in at least two cell cultures of primate origin.

Progress and Summary:

With the exception of lymphocytic choriomeningitis (LCM) virus¹ no studies appear to have been directed to determination of the sensitivity of arenaviruses to the antiviral activity of interferon (IF). Several members of the Tacaribe-complex plaqued quite visibly on monolayers of African green monkey kidney cells, BS-C-1 (cells more sensitive to human fibroblast IF than Vero cells). This fact determined the cell type initially used for assaying arenavirus sensitivity to IF. Interferon for sensitivity studies was prepared from human skin cells, Detroit 532, by an IF priming and superinduction procedure with poly I:C. Assay of IF activity was principally by plaque reduction, using a 50% endpoint, obtained by probit transformation. The IF sensitivity of the arenavirus member studied was compared to the IF sensitivity of vesicular stomatitis virus (VSV) in simultaneous titrations. Results of this procedure are shown in Table I, and reveal that no arenavirus member examined was as IF-sensitive as VSV. BS-C-1 cells required treatment with 10-630 times more human IF to inhibit the various arenaviruses than to inhibit the commonly used challenge virus, VSV.

TABLE I. ARENAVIRUS SENSITIVITY TO HUMAN INTERFERON AS ASSAYED IN MONKEY CELLS (BS-C-1)

CHALLENGE VIRUS	FOLD INCREASE IN INTERFERON NEEDED TO INHIBIT ARENAVIRUS OVER VSV
Parana, Tamiami, Tacaribe	7-10
Amapari, Machupo, Pichinde	50-100
Lymphocytic choriomeningitis, Latino	>100-630

Since the type species, LCM virus, proved relatively IF-insensitive, its response to IF was also examined by a yield-reduction assay. The IF preparation used had titered 25-50,000 against VSV by plaque-reduction

in monkey cells. The result of this assay is shown in Table II. Defining the IF titer in this assay procedure as the reciprocal of the dilution of IF which reduced LCM virus yield by 0.5 log₁₀ from the LCM virus yield obtained from monkey cells treated with only maintenance medium ($10^{5.2}$) placed the IF titer against LCM virus at 320 ($10^{5.2} - 10^{0.5} = 10^{4.7}$). This result also suggests the

TABLE II. DETERMINATION OF SENSITIVITY OF LCM VIRUS (ARMSTRONG) TO HUMAN INTERFERON BY A YIELD-REDUCTION PROCEDURE CARRIED OUT IN CELLS OF MONKEY ORIGIN (BS-C-1)

IF DILUTION	LOG ₁₀ OF IF DILUTION	LOG ₁₀ LCM YIELD (PFU/ml) IN HUMAN SKIN CELLS
1/10	- 1.0	4.1
1/32	- 1.5	4.3
1/100	- 2.0	4.6
1/320	- 2.5	4.7
None	0.0	5.2

relative insensitivity of this virus to human IF in BS-C-1 cells by an assay procedure which is at least as sensitive for IF assay as plaque-reduction.

Because arenavirus members may display a different susceptibility pattern to IF relative to VSV on human cells compared to monkey cells,² arenavirus IF sensitivity was also tested on human skin cells. These results appear in Table III, and reveal also that one of the arenaviruses examined proved to be as sensitive to IF as VSV. It can also be seen that all 3 arenaviruses appeared to be more uniformly sensitive to IF in human cells in contrast to their varied IF susceptibilities found in the BS-C-1 assay system.

TABLE III. ARENAVIRUS SENSITIVITY TO HUMAN INTERFERON AS ASSAYED IN HUMAN CELLS (DETROIT 532)

CHALLENGE VIRUSES	FOLD INCREASE IN INTERFERON NEEDED TO INHIBIT ARENAVIRUS OVER VSV
Parana	4-8
LCM	9
Pichinde	10

Explanations for the lessened sensitivity of arenavirus to IF compared to VSV are conjectural. One explanation might include the possibility that the decay rate³ for the IF induced antiviral state is more rapid in BS-C-1 cells than in human skin cells, and more rapid in BS-C-1 cells using the least IF-sensitive arenavirus members. Since host cell RNA species have been shown to reverse IF-induced antiviral activity,⁴ the presence of host cell-like 4-6S transfer RNA species within arenavirus virions or perhaps within host cells infected with arenaviruses may partially account for reversal of IF intrinsic antiviral protein.

When studying sensitivity of viruses to IF, it seems traditional to also consider IF induction by these viruses. Because IF induction by viruses is dependent upon variables of host cell and virus, it was important to examine the ability of arenaviruses to induce IF in at least 2 different cell cultures. It is clear that LCM virus does not induce synthesis of IF in mouse cell cultures.⁵ Interferon and arenavirus titers in culture fluids of monkey and human skin cells were measured after inoculation with several arenaviruses. Table IV shows that only minimal to undetectable levels of IF were induced following 72 hr arenavirus infection of monkey cells. Moreover, when human cells treated with low dilutions of culture fluids from arenavirus were grown in BS-C-1 infected cells, all processed fluids appeared to enhance VSV plaques over VSV plaques enumerated on human cells treated with maintenance medium. In a separate experiment, LCM virus inoculated onto BS-C-1 cells induced 10 international reference units (IRU) of IF as assayed on BS-C-1 cells against VSV.

TABLE IV. LACK OF EFFICIENT INTERFERON PRODUCTION IN BS-C-1 CELLS IN ROLLER BOTTLES AFTER INFECTION WITH SEVERAL ARENAVIRUSES (MOI=0.1)

INDUCING ARENAVIRUS	PROCESSED FLUIDS (dilution)	% CHANGE IN VSV CHALLENGE PLAQUES ^a AFTER PRETREATMENT OF IF BIOASSAY CELLS		ARENAVIRUS RELICATION PFU/ml (\log_{10})
		Human (CCL54)	Monkey (BS-C-1)	
Parana	1/4	+60	-35	7.1
	1/8	+49	- 6	
Pichinde	1/4	+13	-34	7.5
	1/8	+43	-44	
Tamiami	1/4	+46	-54	6.5
	1/8	+97	-51	
Tacaribe	1/4	+72	-31	5.5
	1/8	+128	-39	

^aAs compared to VSV plaques in presence of normal maintenance medium.

Induction of IF production in human skin cells, and concomitant relicative titers for the arenavirus members are shown in Table V. Interferon production in human cells after 72 hr of arenavirus infection increased in order from Tacaribe, Tamiami, Pichinde, Parana, to LCM virus. The antiviral activity was

TABLE V. INTERFERON PRODUCTION BY SELECTED ARENAVIRUSES IN HUMAN SKIN CELLS
(MOI=1.0)

INDUCING ARENAVIRUS	INTERFERON TITERS (IRU) VS. VSV (PR ₅₀) IN:		ARENAVIRUS REPLICATION \log_{10} PFU/ml
	Human cells	Monkey cells	
LCM	630	180	5.4
Parana	450	130	5.5
Pichinde	25	7	5.5
Tamiami	20	4	4.7
Tacaribe	6	3	4.7

characterized as being acid-stable (pH 2.0), nondialyzable, also inhibitory for attenuated VEE(TC-83), and Semliki Forest virus with dosage responses linear over a 1-1.5 \log_{10} range of dilutions. The ratio of antiviral resistance conferred upon human and monkey cells is characteristic of the antiviral resistance induced in these cells by human fibroblast IF prepared by IF priming and superinduction with poly I:C. Interferons in LCM, Parana, and Pichinde culture fluids were not sedimented by 35,000 x g for 3 hr as assayed in BS-C-1 cells against VSV, nor did these interferons confer antiviral resistance to L-929 cells against VSV. No IF activity was demonstrable with culture fluids obtained from mock-infected human cells.

Thus, several arenaviruses were found relatively less sensitive than VSV to a human IF in a monkey and human cell line. Arenaviruses appeared not to induce significant amounts of IF in monkey cells, but induced detectable quantities in a human cell line. The results of the IF induction experiments suggest that arenavirus members selected are relatively inefficient IF inducers, and that IF probably plays a minor role in resistance of primates to arenavirus infections.

This technical support plan is terminated.

LITERATURE CITED

1. Mims, C. A., and T. P. Subrahmanyam. 1966. Immunofluorescence study of the mechanism of resistance to superinfection in mice carrying the lymphocytic choriomeningitis virus. J. Path. Bacteriol. 91:403-415.

2. Stewart, II, W. E., and R. Z. Lockart, Jr. 1970. Relative antiviral resistance induced in homologous and heterologous cells by cross-reacting interferons. *J. Virol.* 6:795-799.
3. Hallum, J. V., H. R. Thacore, and J. S. Youngner. 1970. Factors affecting the sensitivity of different viruses to interferon. *J. Virol.* 6:156-162.
4. Revel, M., J. Content, A. Zilberstein, B. Lebleu, E. Falcoff, and R. Falcoff. 1975. Mechanism of interferon-induced block in mRNA translation. *Persp. Virol.* 9:233-248.
5. Lehmann-Grube, F. 1967. A carrier state of lymphocytic chorio-meningitis virus in L cell cultures. *Nature* 213:770-773.

TSP-20

AUTOMATED BIOCHEMICAL ANALYSIS

Biochemical Quantitative Techniques - Karen A. Bostian, B.S.

Amino Acid Analyses - Richard E. Dinterman, B.S.

Trace Metal Analyses - Edward C. Hauer, B.S.

LTC M. V. Kaminski, Jr., Coordinator

Physical Sciences Division

In accordance with USAMRIID Memorandum 70-3, dtd 18 Aug 75, technical support projects previously numbered TSP-01, 02, and 14 are now combined under a single coordinator.

Man-hours per week authorized to sustain this project is 240. A summarized report of the activities of each support laboratory is as follows:

Automated Biochemical Techniques

Objective:

Develop, or find and utilize reliable methods to quantitate concentrations of various metabolites in fluids including blood, urine, feces and tissue extracts from both man and laboratory animals utilizing an AutoAnalyzer system (Technicon).

Progress and Summary:

During the past fiscal year 91,402 samples were processed with an average requirement of 140 man-hours per week. This represents a marked increase in productivity despite a reduction in personnel. (FY 1975: 56,765 samples, 155 man-hours/week). This increase however is due to voluntary commitment of personal time, a decreased application for leave, and continuous utilization of equipment.

FY 1976 samples by divisions:

Physical Sciences	49%
Pathology	37%
Bacteriology	6%
Rickettsiology	2.8%
Animal Resources	2.8%
Virology	2%
Animal Assessment	0.2%
Aerobiology	0.2%

Of the 91,420 procedures performed special handling was required as follows:

47% completely automated systems

17% required preparation of Somogyi filtrate

14% required extraction of lipids

12% required special dilutions, standarization and monitoring for analysis of specific proteins

6% required special dilutions

4% required special reagents, standardization and equipment. This includes analysis for enzymes, T₄, TIBC, and Kjeldahl nitrogen.

Technical Advances:

1. An automated system for serum Fe and total Fe-binding capacity, which is accurate, free of error secondary to hemolysis and produces consistent results, was initiated using the Technicon AutoAnalyzer II system.

2. To define the mechanism of action of bacterial exotoxins on cell cultures, an automated system for Lowry protein was developed.

3. Automated systems were also modified utilizing immunoprecipitin techniques for rat albumin, transferrin, γ -globulin, fibrinogen, C₃ and α_2 -macroglobulin as well as albumin for the hamster, guinea pig and monkey model.

4. Application was made of AutoAnalyzer phosphorus analysis to monitor column effluent for Virology Division.

5. Special micro-methods were adapted to the AutoAnalyzer to use in the study of RMSF in monkeys for analysis of serum concentrations of triglycerides, free fatty acids, cholesterol, albumin, and urea nitrogen.

6. An automated system to determine glycerol concentrations was put into routine operation.

7. Mr. Billy Blackburn was instrumental in developing new programs for analysis of data on the Wang computer.

Amino Acid Analysis:

Objective:

Develop and utilize rapid, reliable methods for quantitation of amino acids in various biological fluids using the Technicon Amino Acid Analyzer.

Progress and Summary:

During the period covered by this report a total of 612 analyses were performed requiring 60 man-hours/wk. This represents an increase in productivity with essentially no increase in technical assistance. (FY 1975: 552 analyses). Machine time required for specific analyses is as follows:

Physiological amino acids	5½ hr
Hydrolysate amino acids	4 hr
Phenylalanine and tyrosine	90 min
Hydroxyproline and 3-methylhistidine	3 hr
Alanine	2 hr

The type, number and source of analyses performed are as follows:

	<u>Division</u>
Physiological (33 amino acids)	393
Hydrolysate (18 amino acids)	117
Phenylalanine and tyrosine	24
Hydroxyproline and 3-methylhistidine	54
Alanine	24
	Physical Sciences
	45% Pathology
	32% Virology
	23% Bacteriology
	Physical Sciences
	Physical Sciences
	Physical Sciences

Technical Advances:

1. An automated system for quantitating S-carboxymethyl cysteine was developed. It was discovered that a 0.2 N sodium buffer (pH 3.25) produces complete separation of S-carboxymethyl cysteine in approximately 45 min.

2. An automated system for fraction collection of phenylalanine was developed using the first column of the TSM Analyzer. The sample can then be transferred to a scintillation counter to quantitate ^{14}C -labeled phenylalanine.

Trace Metal Analysis:

Objective:

To provide rapid and reliable methods of analysis of trace metals in various biological specimens.

Progress and Summary:

A total of 25,294 analyses of serum, plasma, tissues, urine or medium were performed for Zn, Cu, Fe, Ca, Mg, Cd, Mn, Cr, Li and ceruloplasmin.

Sample turn-around time was recently added as a new criterion to evaluate the effectiveness of the trace metal service. It was determined that 78% of all analyses were reported out within 3 days of receipt. For the last month of the 4th quarter, the rate was brought up to 97.5%. This section requires 80 man-hours/wk.

Mr. Hauer was additionally instrumental in generating the following programs for the Wang computer for several principal investigators of Physical Sciences Division: 12-column analysis of variance including calculation of mean, SD, SE, least significant difference and mean differences; linear regression analyses with slope confidence limits and y to x conversions with statistics on x; frequency table and histogram; 12-column statistics with confidence limits.

Technical Advances:

Methods were developed during FY 75 for determination of metals in tissues using either low temperature ashing or tetramethyl-ammonium hydroxide techniques of sample preparation.

TSP-21
AUTOMATIC DATA PROCESSING SUPPORT FOR USAMRIID
Thomas J. Powers, CPT, MSC
Administrative Division

Objective:

Automatic data processing technical support consists of maintaining the capability of utilizing computers and calculators to process repetitive data generated by work units within USAMRIID.

Progress and Summary:

Computer support over the past year has shown a significant improvement. Initial efforts were to restore support to previous Data Systems Agency support levels. This took approximately 2 months, and culminated in the establishment of a reliable terminal hookup to the CDC 3500 at WRAIR. Since that time, further enhancements have been made to expand our capabilities. An emulator, the Univac 1004 package, was acquired from Data 100 Corporation to allow us to direct-dial the Univac 1108 Computer Center at National Bureau of Standards. This has provided us with a systems analysis package for mathematical modeling, a sophisticated library of statistical subroutines, and the use of interactive Fortran, Cobol and XBASIC. A further improvement in terminal operation was achieved with the replacement of our old card input device with a faster, more reliable card reader. Our terminal has demonstrated usefulness not only in an on-line mode, but also in an off-line mode, producing mailing labels, test tube identification labels and card lists for input verification. In summary, the installation of our Remote Job Entry computer terminal has improved computer support beyond any previous support level, and continued improvement can be expected.

Current technical support tasks are:

1. Immunization - the large immunization data base has been converted from the old CDC 3300 computer to the new system at WRAIR. The data were reorganized to expand overall system capabilities. Retrievals were made referencing information collected over the past 2 yr on tularemia and vaccine lots. The full implementation of the system is expected shortly.

2. Library - the library system provides an accounting mechanism for all volumes in the USAMRIID Library. The system had been hampered in the past by poor programming, lack of an on-line data base and lack of a timely update procedure. All previous shortcomings were eliminated with the implementation of a new library system. The new system produces periodic division listings, annual library master listings, and monthly update transaction listings to validate library activity during each month. The new system should stabilize applications programming in this technical support area.

3. Paper Tape - this system consists of computer programs used to process paper tape produced by the β and γ liquid scintillation counters. During the year, approximately 100 paper tapes were processed. This represents a significant decrease in activity in this area even though our ability to return processed output to the investigator has significantly improved. Changes in investigative personnel and research emphasis have been the principal reasons for the decreased activity.

4. Registry of Infectious Organisms - a master file of all infectious organisms currently used at USAMRIID is continually maintained to reflect current usage. The computer master file is periodically sorted down and printed by organism, division, location of use, and registry number. These printed reports are forwarded to the Safety Officer for appropriate distribution.

5. Label Printing - the off-line use of our computer terminal to produce labels for various identification requirements has significantly improved our ability to respond to label requests. Usually a request for label generation can be satisfied the same day. Approximately 2,400 labels were produced during the past year.

6. Calculators - programming support has been provided to requesting divisions for solutions to problems within the capabilities of programmable calculators. Additional support in this area was achieved through the acquisition of about 100 MONROE 1860 programs that provide solutions to a variety of small mathematical and statistical problems.

7. Probit Analysis - probit analyses for the determination of LD₅₀ were processed for investigators on an as-required basis. Both probit programs are operational on the CDC 3500 at WRAIR.

8. Renal Function - the data base of renal function data was converted to a new format to facilitate further statistical analysis. An update program was written to improve the processing of changes and additions to the data base. Program modification of the old programs was accomplished to accommodate the new formats.

9. Cardiovascular Data - the system of routines to process cardiovascular data was converted and debugged to run on the CDC 3500 at WRAIR. The backlog of data that had accumulated was processed and the results returned to the physiology laboratory.

10. Pathology Accessions - an information retrieval system for pathology accessions is being designed. Initial efforts to develop an input coding system have concluded and new accessions are being coded for conversion to the automated data base. Data base conversion and system design and development are scheduled to run concurrently. The system, once complete, should prove to be a valuable research tool.

TSP-22

RESEARCH SEROLOGY AND VIROLOGICAL SUPPORT SERVICES

Joseph A. Mangiafico, M.P.H., Bacteriology Division (Part A)
Helen H. Ramsburg, A.B., Virology Division (Part B)

Objectives:

(1) To provide centralized services for serological evaluation of antigen and/or antibody content in biological specimens derived from approved work units; (2) to conduct research for the development, standardization and evaluation of new serological procedures or the modification of established serological procedures in support of specific USAMRIID requirements; and (3) to provide virological support service for all studies carried out in volunteers and/or involving efficacy, safety and epidemiological assessment of vaccines in humans.

Progress and Summary:Part A - Research Serology

Research Serology Section was converted to a "hot suite" operation in order to employ infectious antigens. The use of these antigens expanded the capabilities of the Section, particularly in the conduct of plaque reduction serum neutralization tests (PRSN) for selected arboviruses. Antigens for use in the PRSN were prepared with YF (Asibi strain), EEE (PE-6 strain), WEE (B-11 strain) and VEE (Trinidad strain) viruses. In addition, inactivated antigens for 15 group B arboviruses were received for routine testing of sera for HI antibody to these group B arboviruses.

Passive HA tests were also developed for detecting and measuring serum antibody to Staphylococcus aureus exfoliative toxin and Pseudomonas aeruginosa exotoxin A using BDB as a coupling agent and sheep RBC as the carrier. A similar test is being evaluated for Salmonella typhimurium.

A CF test for detecting and measuring serum antibody to Mycoplasma pneumoniae has been added to the routine test group.

Research initiated last year to evaluate the feasibility of employing chemically-fixed gander RBC to detect and measure antibody to selected group A arboviruses by the HI test was discontinued during this year. Fixed RBC were found to be insensitive and nonspecific in measuring HI antibody to VEE virus, although good results were obtained with EEE and VEE viruses.

During this year, Research Serology conducted approximately 16,028 serological tests in support of USAMRIID work projects. The types and number of serological tests performed by division are shown in Table I. The number of tests conducted by Research Serology during FY 76 has increased by 85% over the number of tests conducted in FY 75.

TABLE I. SEROLOGIC PROCEDURES PERFORMED BY RESEARCH SEROLOGY - FY 76

DIVISION	TYPE AND NO. OF TESTS						TOTALS BY DIVISION
	Agglutinin	CF	HA	HI	Oudin	PRSN	
Aerobiology	443	24	289				756
Animal Assessment		91	1949				2040
Animal Resources		1088	1945				3033
Bacteriology		92					92
Medical	607	418	139	2174		904	4242
Pathology			154		1094		1248
Physical Sciences	83		8			45	136
Rickettsiology		22	3	30		64	119
Virology	4	12	135	2869		69	3089
Other	334	91	156	533		159	1273
TOTALS	1028	986	1890	9789	1094	1241	16,028

Part B. Virological Support Services

Standard PRSN tests¹ were performed to measure the level and duration of homologous and/or heterologous neutralizing antibodies formed against live, attenuated VEE vaccine (TC-83), YF vaccine (17-D), dengue-2 (Den-2) vaccine or against formalin-inactivated VEE, EEE, or WEE vaccines. In addition, sera from at-risk personnel and monkeys exposed to Den-1, Den-2, JBE, Langat, West Nile or YF group B arboviruses were tested for the presence of neutralizing antibodies.² Tests are summarized in Table II.

Tests conducted to determine the optimal system for virus preparation and the optimal tissue culture system for measuring PRSN antibody in serum from 3 mammalian species are included in Table II. These studies demonstrated that the sensitivity of the PRSN test was markedly affected by the species of cells used for virus production, or by the cell strain used for detection of antibody activity. Evaluation is still in progress, and results of these studies will be reported at a later date.

A PRSN test was standardized for representative VEE subgroup strains: MF-8 (IB), V-198 (IC), 3880 (ID), Mena II (IE), Fe-3-7c (II), Mucambo (III), and Pixuna (IV). The number of tests performed to measure the level and duration of neutralizing antibodies formed against these strains in response to vaccination with live or formalin-inactivated TC-83 vaccine are presented in Table III.

TABLE II. PRN TESTS PERFORMED WITH GROUP A AND B ARBOVIRUSES - FY 76

INVESTIGATOR SERUM (Division)	TESTED	NO. OF SAMPLES TESTED									
		VEE		EEE	WEE	Den-1	Den-2	JBE	Langat	WN	YF
		TC-83 Trin.									
Burke (Medical)	Human	92	133	95	99						2
Cole (Virology)	Monkey										
	Rabbit		3	3	3						
	Rat		69	51	53						
Eddy (Virology)	Human	18				7	8	2	17		25
	Monkey		23					34			
Edelman (Virology)	Monkey	8	8	8	8	28	28	23	28	24	
Harrington (AA)	Human								12		
Ramsburg (Virology)	Horse		101								
	Human		204								
	Rat		101								
Sammons (Rickett.)	Monkey		6			6	6	6	6	6	

TABLE III. PRN TESTS PERFORMED WITH VEE SUBGROUP STRAINS - FY 76

INVESTIGATOR SERUM (Division)	TESTED	NO. OF SAMPLES TESTED							
		MF-8 (IB)	V-198 (IC)	3880 (IC)	Mena II (IE)	Fe-3-7c (II)	Mucambo (III)	Pixuna (IV)	
Burke (Medical)	Human	63	62	62	62	62	62	62	60
Edelman Virology	Monkey	8	8	8	8	8	8	8	8

Standard PRSN tests were employed in cross-protection studies for detection of homologous and heterologous antibodies after infections with arenaviruses. In addition, at-risk personnel were tested for the presence of arena virus neutralizing antibodies. These tests are summarized in Table IV. The standard procedure was found to be less sensitive than mouse neutralization for measuring neutralizing antibody against some arenaviruses. Evaluation of tissue culture systems is in progress to develop a more sensitive test. Since addition of complement to the test system failed to increase sensitivity, future studies will be concerned with testing purified or cloned virus.

TABLE IV. PRN TESTS PERFORMED WITH ARENAVIRUSES - FY 76

INVESTIGATOR (Division)	SERUM TESTED	NO. OF SAMPLES TESTED				
		Amapari	Latino	Parana	Pichinde	Tacaribe
Eddy (Virology)	Human	78	75	75	73	77
	Monkey	20		16	12	222
Rosato (Virology)	Guinea pig					6
	Hamster					3
	Mouse			34		46
	Rat					9

Virological support services performed in support of vaccine studies are summarized in Table V.

TABLE V. VIROLOGICAL SUPPORT SERVICES - FY 76

INVESTIGATOR (Division)	SPECIMEN TESTED	NO. OF SAMPLES TESTED		
		Virus Preparation	Virus Titration	Virus Characterization
Eddy (Virology)	Horse sera	3	6	In process ^a
	Hamster brain	2	4	
	Horse brain	1	2	
Edelman (Virology)	Vaccine	3	18	18 ^b
Metzger (Pathology)	Tissue culture fluids		76	None required
Mangiafico (Bacteriology)	See virus supplied for preparing stock viruses for use in VEE, EEE, WEE, and YF PRSN tests.			

^a Suspected VEE virus isolates from Colombia are being confirmed by neutralizing antibody tests.

^b Vaccine virus recovered from vaccinees was characterized for plaque size.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases, 1 July 1973. Annual Progress Report, FY 1973, p. 460-462. Fort Detrick, Frederick, MD.
2. U. S. Army Medical Research Institute of Infectious Diseases, 1 July 1974. Annual Progress Report, FY 1974, p. 449-453. Fort Detrick, Frederick, MD.

TSP-23
TECHNICAL SUPPORT FOR CELL CULTURE PREPARATION
 John P. Kondig
 Virology Division

Objective:

To provide a centralized facility for the preparation or acquisition of cell cultures, media reagents and related materials, including viral vaccine substrates for use by investigators of the Institute.

Progress and Summary:

During the past year the cell culture preparation and supply laboratory has produced approximately 6,000 L of tissue culture medium and balanced salt solutions. It has also provided a total of 30 cell lines, strains, or primary cells in the various quantities listed in Table I.

TABLE I. CELL CULTURES PRODUCED BY DIVISION AND INVESTIGATOR

DIVISION AND INVESTIGATOR	NUMBERS				
	Roller bottles	T-75 Flasks ^a	T-25 Flasks ^a	6-well Plates	Tubes ^a
<u>Aerobiology</u>					
Kishimoto	120				72
Walker		10			
<u>Animal Assessment</u>					
Harrington	22	164		5200	80
Houston	41	30		1980	3765
Stephens			166		2899
Heard	32		6		
<u>Bacteriology</u>					
Mangiafico		10	600	6559	
Canonico			288		
<u>Pathology</u>					
Metzger		52			80
Middlebrook	50	1063			414
Wing		77			
Andron	39				
White					12
Anderson	6				
<u>Physical Sciences</u>					
Zenser		34	590		
Mapes	20				

TABLE I. CELL CULTURES PRODUCED BY DIVISION AND INVESTIGATOR (Continued)

DIVISION AND INVESTIGATOR	NUMBERS					
	Roller bottles	T-75 Flasks ^a	T-25 Flasks ^a	6-well Plates	Tubes ^a	24-well Plates
<u>Rickettsiology</u>						
Bagley	22					836
Johnson			10,210			
Kenyon	446		2,510			
Oster	370					
Pedersen	390		390			
Williams	50		485			
Wachter	40					
Rudczynski	99	35	585			
<u>Virology</u>						
Cole		240	11,610			
Eddy	29	657	1,645	1,175	64	1,029
Gangemi	1,707	111	235	1,015	36	
Jahrling	764	71	120	9,450	144	
Levitt		22	40	3,345	35	
Luscri	76	112	4,457			
Ramsburg	4	143	1,890	6,420		144
Rosato	125	23	520	1,256		
Wear			1,865			
Seed	1,635	8,781				
Total seed	4,452	2,854	38,212	36,400	1,279	8,331
Total + seed	6,087	11,635				

^aOr equivalent.

TSP-24

LABORATORY ANIMAL SUPPORT
 Robert L. Hickman, MAJ, VC
 Animal Resources Division

Objective:

To provide comprehensive laboratory animal support to include (a) animal health care and evaluation; (b) complete surgical service for laboratory animals; and (c) maintenance of a source of animal blood and sera.

Progress and Summary:

A total of 99,740 laboratory animals were purchased from 15 commercial and 4 noncommercial sources. Numbers of animal requisitions, animal deliveries, and animals issued to each division are presented in Tables I and II.

TABLE I. ANIMAL PROCESSED AND RECEIVED

DIVISION	ANIMALS REQUISITIONS/ANIMAL DELIVERIES BY QUARTERS				
	FY 75-4	FY 76-1	FY 76-2	FY 76-3	Total
Aerobiology	25/ 31	21/ 24	20/ 22	21/ 22	87/ 99
Animal Assessment	31/ 31	47/ 50	23/ 23	31/ 31	132/ 135
Animal Resources	4/ 4	8/ 8	7/ 7	8/ 8	27/ 27
Bacteriology	17/ 17	13/ 17	18/ 18	18/ 33	66/ 85
Pathology	15/ 22	12/ 13	10/ 14	8/ 18	45/ 67
Physical Sciences	65/169	42/ 91	43/102	55/138	205/ 500
Rickettsiology	9/ 11	11/ 16	11/ 11	12/ 13	43/ 51
Virology	23/ 41	19/ 76	18/ 26	22/ 42	82/ 185
TOTALS	189/326	173/295	150/223	175/305	687/1,149

Quality control evaluation of new animals and examination of issued animals continued to provide evidence of health problems in laboratory animals used at this Institute. Minimal to moderate respiratory diseases are the most common problem in new animals affecting 14% of the mice, 44% of the rats, 20% of the hamsters and 75% of the guinea pigs purchased. The following are specific diseases identified one or more times in different species during the year.

Mice: Chronic respiratory disease (Mycoplasma spp.); acute and chronic parainfluenza (Sendai virus); Tyzzer's disease (Bacillus piliformis); oxyuriasis.

Rats: Chronic respiratory disease (Mycoplasma spp.); acute salivary gland infection (sialodacryoadenitis virus).

Guinea Pigs: Acute and chronic pneumonia and lymphadenitis (Streptococcus spp.).

TABLE II. ANIMALS ISSUED

DIVISION	NO. BY SPECIES					
	Mice	Rats	Hamsters	Guinea pigs	Rabbits	Monkeys
Aerobiology ^a	14,319	1,623	650	366	0	57
Animal Assessment ^b	18,875	1,850	10	91	199	412
Animal Resources ^c	0	0	300	103	0	29
Bacteriology	16,685	751	129	138	21	5
Pathology	2,129	226	0	150	73	91
Physical Sciences	5,135	21,070	0	590	2,334	84
Rickettsiology ^a	1,360	0	0	1,187	18	29
Virology ^a	4,300	1,446	2,261	457	10	113
TOTALS	62,803	26,966	3,350	3,082	2,655	820

^a Other rodents: Aerobiology, 117; Rickettsiology, 93; Virology, 186.

^b 4 Burros, 16 swine.

^c 6 Chickens.

Rabbits: Coccidiosis; pasteurellosis; ringworm, otitis externa (Psoroptes cuniculi).

Monkeys: Acute enteritis (Shigella spp.); chronic bacterial enteritis (various spp.).

All macaque monkeys (Macaca mulatta and M. fascicularis) were tested on arrival and each quarter thereafter for tuberculosis. No reactors were identified and no suspicious lesions were found on routine necropsy of experimental animals. A total of 3,070 serologic screening tests were performed at the request of various investigators. Results are presented in Table III.

A total of 18 monkeys, 33 sheep, 23 goats, 12 burros, 1 horse, 8 chickens, 3 geese and 26 guinea pigs were being maintained as blood and serum donors at the end of the reporting period. Additional monkey blood was obtained from colony animals; additional chicken and goose blood was purchased. The volume of blood collected or purchased by species for each of the using divisions is presented in Table IV.

Complete laboratory animal surgical support was provided to several investigators during the year. The number of surgical procedures performed is listed in Table V. In addition, surgical assistance and radiologic support was provided as requested.

TABLE III. RESULTS OF SEROLOGIC SCREENING^a OF NONHUMAN PRIMATES

RECIPROCAL TITER	NO. POSITIVE									
	SE (n = 330)			Dengue ^b			WN ^b	JBE ^c	SLE ^d	YF
	A	B	C	I	II	III				
10	18	28	34	9	8	10	27	6	19	6
20	23	17	24	22	20	21	15	12	28	6
40	1	16	12	10	7	7	8	1	11	9
80	1	7	23	7	2	3	6	5	5	4
160	2	4	13		4	1	1		1	4
320	1	5	14		1					
640		2	18							
> 640			13							
% POSITIVE	14	24	46	17	15	15	20	8	22	12

^aInfluenza, 52 tested, all < 1:10.^bn = 288.^cn = 292.^dn = 290.

TABLE IV. VOLUMES AND TYPES OF BLOOD SUPPLIED

ANIMAL	ml BLOOD BY DIVISION									TOTAL ml
	Aero	AA	AR	Bact	Path	PS	Rick	Virol	Serol	
Monkey	600	1,825	140	3,331	1,330	150	1,080	450	40	8,946
Sheep				10,625	100	35,925	645	4,775		52,070
Goat				1,300	305			5		1,610
Goose				40				5	940	985
Chicken	345				5			15		365
Horse				10	1,540					1,550
Burro				500	5					505
G. pig	592	20		20	5			60		695
Rabbit				617				10		627

TABLE V. SURGICAL PROCEDURES PERFORMED ON LABORATORY ANIMALS

SURGICAL PROCEDURES	NO. PERFORMED
Catheterizations	
Femoral artery	111
Femoral vein	167
Internal jugular	49
External jugular	48
Carotid artery	68
Renal vein	29
Portal vein	2
Left ventricle	24
Saphenous vein	5
Thoracic duct	3
Gut loop formation	23
Cardiac transducer implant	3
Gastrotomy	2
Splenectomy	7
Pancreatectomy	2
Biopsy	28
Vessel flow probes	2
Face mask procedure	5
Colony care	22
TOTAL	600

Publications:

1. Moe, J. B., J. B. White, W. P. Czajkowski, and J. L. Stookey. 1975. Myxocarcoma in a young rhesus monkey. *Vet. Pathol.* 12:6-12.
2. Faulkner, R. T., D. G. Harrington, M. L. Sammons, D. E. Hilmas, and L. Sammons. 1976. A case of esophageal foreign body with mediastinal abscess formation in a dog. *J. Am. Animal Hosp. Ass.* 12:70-76.
3. Faulkner, R. T., W. P. Czajkowski, E. J. Rayfield, and R. L. Hickman. 1976. Technique for portal catheterization in rhesus monkeys (*Macaca mulatta*). *Am. J. Vet. Res.* 37:473-475.
4. Faulkner, R. T., W. P. Czajkowski, D. G. Harrington, F. E. Chapple, and R. L. Hickman. 1976. Internal fixation of humeral fractures in rhesus monkeys (*Macaca mulatta*). *Vet. Med./Small Anim. Clinician* 71:643-647.

TSP-25
BACTERIAL CULTURE SERVICE AND MEDIA SUPPLY
 Wallace G. Fee (Part A)
 William E. Kline (Part B)
 Bacteriology Division

Objective:

Part A. To provide a centralized facility to maintain a stock culture collection of bacterial strains with complete records of source and cultural history and to supply well characterized bacterial suspensions for use in approved work units within the Institute.

Progress and Summary:

Bacterial culture service was approved as TSP-15 on 26 September 1974. The Culture Service and Media Supply were assigned to TSP-25 on 3 February 1976. Services provided USAMRIID investigators are listed in Table I.

TABLE I. SERVICES PROVIDED DURING FY 76

DIVISION AND INVESTIGATOR	ORGANISM	STRAIN	NO. SAMPLES
<u>Physical Sciences</u>			
Neufeld and Pace	<u>Streptococcus pneumoniae</u>	Type I, A-5	100
	<u>Francisella tularensis</u>	LVS	5
	<u>Salmonella typhimurium</u>	USAMRIID M.I.T.	4
Dinterman	<u>S. pneumoniae</u>	Serotype I, A-5	84
Powanda	<u>Staphylococcus aureus</u>	ATCC #14458	11
	<u>S. pneumoniae</u>	Serotype I, A-5	2
	<u>F. tularensis</u>	LVS	5
	<u>S. typhimurium</u>	USAMRIID M.I.T.	1
	<u>Staphylococcus epidermidis</u>	ATCC #14990	1
Kaufmann	<u>S. typhimurium</u>	USAMRIID M.I.T.	4
Mapes	<u>S. aureus</u>	ATCC #14458	1
Thompson	<u>S. pneumoniae</u>	Type I, A-5	5
Petrella	<u>S. pneumoniae</u>	Type I, A-5	20
	<u>S. typhimurium</u>	USAMRIID M.I.T.	3
George	<u>S. pneumoniae</u>	Serotype I, A-5	2
Strobel	<u>S. pneumoniae</u>	Serotype I, A-5	4
Beall	<u>S. pneumoniae</u>	Serotype I, A-5	1
Sullivan	<u>S. pneumoniae</u>	Serotype I, A-5	3

TABLE I. SERVICES PROVIDED DURING FY 76 (Continued)

DIVISION AND INVESTIGATOR	ORGANISM	STRAIN	NO. SAMPLES
<u>Pathology</u> Wing	<u>S. typhimurium</u>	USAMRIID M.I.T.	1
<u>Aerobiology</u> Berendt	<u>S. pneumoniae</u>	ATCC #6301	8
Dominik	<u>S. pneumoniae</u> <u>F. tularensis</u> <u>S. typhimurium</u> <u>S. aureus</u>	ATCC #6301 LVS USAMRIID M.I.T. ATCC #14458	21 4 3 2
<u>Virology</u> Miller	<u>Escherichia coli</u>	ATCC #11775	1
<u>Bacteriology</u> Fine	<u>S. pneumoniae</u>	ATCC #6301 ATCC #6303 ATCC #6304 ATCC #6307 ATCC #6308 ATCC #6312 ATCC #6314 ATCC #6325	1 1 1 1 1 1 1 1
Yamada	<u>S. typhimurium</u>	USAMRIID M.I.T.	3
Canonico	<u>S. pneumoniae</u> <u>F. tularensis</u> <u>S. aureus</u>	Type I, A-5 LVS ATCC #14458	36 1 1
Eigelsbach	<u>S. aureus</u> <u>Pseudomonas stutzeri</u>	ATCC #14458 ATCC #17588	1 1
H.B. Hawley	<u>S. typhimurium</u> <u>Klebsiella pneumoniae</u> <u>S. pneumoniae</u>	USAMRIID M.I.T. ATCC #10031 ATCC #6301	8 1 1
Rausch	<u>S. typhimurium</u> <u>S. aureus</u>	USAMRIID M.I.T. ATCC #14458	6 5
Janssen	<u>S. pneumoniae</u>	ATCC #6301	6
Skaio	<u>S. pneumoniae</u>	Serotype I, A-5	2
Rill	<u>S. pneumoniae</u>	Serotype I, A-5	4
<u>Animal Assessment</u>			
Heard	<u>S. typhimurium</u>	USAMRIID M.I.T.	4
Houston	<u>S. typhimurium</u>	USAMRIID M.I.T.	2
Pettit	<u>S. pneumoniae</u>	ATCC #6301	2
VA Hospital Lexington, KY Coonrod	<u>S. pneumoniae</u>	ATCC #6308	1

Part B. To provide centralized facilities for preparation of standardized lots of culture media, solutions and reagents for approved research within the Institute and for clinical laboratories at Fort Detrick, Fort Ritchie and Camp David.

Progress and Summary:

The following media, reagents, etc., were prepared by Media Section during FY 1976 for investigators in Aerobiology, Animal Assessment, Bacteriology, Pathology, Physical Sciences, Rickettsiology, and Virology Divisions and clinical laboratories at Camp David and Fort Ritchie:

PLATES OR TUBES	NO.	BULK MEDIA	LITERS
Agarose	192	Agarose	55.0
Blood agar base and sheep blood	22,079	Alsevers	3.0
Blood agar base plain	12,473	Ammonium chloride buffer	3.0
BI plates	1,120	Alcohol (70%)	6.0
Brain heart infusion agar	596	Borate buffer	6.0
Brain heart infusion broth	5,593	Boric acid solution	2.5
Chocolate agar	360	Brain heart infusion	29.6
Differential agar	130	Casein acid digest	8.0
Eosin methylene-blue agar	1,250	Calcium saline solution	1.0
Earle's 199 medium	100	Cell adjusting diluent	3.0
Formalin solution	25	Dextrose gelatin veronal	8.0
Glucose cysteine blood agar	4,715	Formalin (2%)	0.5
Gelatin saline diluent	1,011	Gelatin phosphate diluent	3.0
Gelatin phosphate diluent	480	Gelatin saline diluent	3.5
Hektoen enteric agar	480	Glycine buffer	1.0
Heart infusion broth	16,016	Gram's iodine	1.6
Lysozyme agar	619	Heart infusion broth	29.5
MacConkey's M	880	Kaolin in borate saline	1.0
Mannitol salt agar	540	Malaria stain	1.0
Modified casein partial hydrolysate	105	Normal saline	69.0
Mueller Hinton	690	NH ₄ Cl solution	5.0
Nutrient agar	549	Nutrient broth	0.5

PLATES OR TUBES	NO.	BULK MEDIA	LITERS
Phosphate buffered saline	1,526	Peptone water	8.0
Physiological saline	12	Phosphate buffered saline	195.9
Sabouraud's dextrose agar	40	Phosphate buffers	51.0
<u>Salmonella-Shigella</u> agar	40	Potassium sulfate solution	2.0
SIM medium	100	Saffranin stain	0.5
Simmon's citrate	50	Sodium nitrate solution	4.0
Sucrose phosphate glutamate buffer	539	Sodium caseinate solution	1.0
Thayer-Martin agar	650	Sodium phosphate solution	6.0
Thioglycollate broth	3,293	Sucrose phosphate glutamate	56.0
Tryptose saline diluent	2,422	Stabilization medium	1.0
Trypticase soy agar	3,759	Thioglycollate	4.0
Trypticase soy broth	170	Tryptose saline	3.0
Urease test medium	50	Tryptose soy agar	2.0
Water distilled (sterile)	1,562	Tryptose soy broth	53.0
	84,160	Tris buffer	1.0
		Veronal buffer	13.0
		Water (distilled)	14,544.0
		Water distilled (sterile)	31.0
		Zinc sulfate	3.0
			15,220.1

APPENDIX B
VOLUNTEER STUDIES

PROTOCOL TITLE AND NO. (No. Volunteers ^a)	COMMENTS AND RESULTS
Acceptability Study of Western Equine Encephalomyelitis Virus Vaccine, Inactivated, Dried, MNLBR 106, Lot 1. Protocol 75-1. (6)	Reported FY 1975 Annual Report. Work Unit 834 02 003.
Evaluation in Volunteers of the Active-Rosette-forming Lymphocyte Test as an Assay for Previous Immunization to Tularemia. Protocol 75-2. (9)	Reported FY 1975 Annual Report. Work Unit 834 02 423.
Persistence of Venezuelan Equine Encephalomyelitis Antibodies following Vaccination with the Live, Attenuated TC-83/3-2 VEE Vaccine. Protocol 75-3. (24 Whitecoats)	VEE antibody persisted in 95% of volunteers 7 and 9 yr after TC-83 vaccination. See Work Unit 834 02 002.
Tuberculin Skin Test Antigen in Man and its Effect on the Active-Rosette-Forming Lymphocyte Test. Protocol 75-4 (9)	Reported FY 1975 Annual Report. Work Unit 834 02 423.
Initial Clinical Evaluation of Rocky Mountain Spotted Fever Vaccine, Formalin-Inactivated Sheila Smith Strain, Chick Embryo Cell Origin, Lot 1, for Safety and Immunogenicity. Protocol 76-1. (6)	Two of 6 individuals developed local reactions. Immunogenicity was satisfactory. See Work Unit 834 02 002.
Acceptability Study of Venezuelan Equine Encephalomyelitis Vaccine, Inactivated, Dried, MNLBR 109, Lot 6-84-1. Protocol 76-2. (18)	The study was begin in April and is incomplete. See Work Unit 834 02 002.

PROTOCOL TITLE AND NO. (No. Volunteers)	COMMENTS AND RESULTS
<p>Rejuvenation and Preservation of <u>Plasmodium vivax</u> (Chesson Strain) and Assessment of Blood Schizontocidal Activity of Mefloquine HCl (WR 142,490). Protocol 76-3. (1 Whitecoat)</p>	<p>The strain was passed and reju- venated successfully. Meflo- quine was an effective drug. See Work Unit 834 02 003.</p>
<p>Immunization of At-Risk (Fort Detrick) Laboratory Workers with Monovalent Influenza A/Swine (A/New Jersey/8/76) Virus Vaccine. Protocol 76-5. (114)</p>	<p>The vaccine was well tolerated. Antibody titers are being evaluated. See Work Unit 834 02 002.</p>
<p>Reactogenicity and Antigenicity of Influenza Virus Vaccines: Bivalent A/Victoria/75 and A/New Jersey/76 and Monovalent B/HongKong/72. Protocol 76-5. (175)</p>	<p>The vaccines were well tolerated. Antibody titers are being eval- uated. See Work Unit 834 02 002.</p>
<p>Reactogenicity of Western Equine Encephalomyelitis Vaccine, Inactivated, Dried, Lot 2-1974. Protocol 76-6. (6)</p>	<p>This lot of WEE vaccine was safe and nonreactogenic. See Work Unit 834 02 002.</p>

^aVolunteers are all staff of USAMRIID unless stated otherwise.

APPENDIX C

U.S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
PROFESSIONAL STAFF MEETINGS

<u>DATE</u>	<u>LECTURER</u>	<u>TITLE OF PRESENTATION</u>
19 Sep 75	Major H. Bradford Hawley, MC Major Tadataka Yamada, MC Bacteriology Division	Disseminated intravascular coagulation in pneumococcal sepsis.
	Major Michael S. Ascher, MC Medical Division	Specific <u>in vitro</u> lymphocyte transformation to Rocky Mountain spotted fever rickettsial antigen.
	Colonel Donald H. Hunter, MSC Bacteriology Division	Further studies on cell-mediated immunity and tularemia.
	Dr. Peter G. Canonico Bacteriology Division	Peroxiwhat? or what you always wanted to know about your organelles.
24 Oct 75	Lt Colonel Gerald A. Eddy, VC Virology Division	Protection against Machupo virus by related arenaviruses of the Tacaribe complex.
	Lt Colonel Robert Edelman, MC Virology Division	Evaluation of attenuated VEE vaccines in man.
	Dr. Peter B. Jahrling, Ph.D. Virology Division	The pivotal role of hepatic reticuloendothelial cells in determining virulence of group A Togaviruses.
	Dr. Neil H. Levitt, Ph.D. Virology Division	Congenital cerebral and ocular malformations induced in Rhesus monkeys by VEE.
5 Dec 75	Dr. Richard H. Kenyon, Ph.D. Rickettsiology Division	Immunological aspects of Rocky Mountain spotted fever vaccines.
	Major Charles N. Oster, MC Rickettsiology Division	Diagnosis of Rocky Mountain spotted fever using radioimmunoassay techniques.
	Dr. Ralph F. Wachter, Ph.D. Rickettsiology Division	Immunological potential of the soluble antigen of <u>Coxiella burnetii</u> .

<u>DATE</u>	<u>LECTURER</u>	<u>TITLE OF PRESENTATION</u>
5 Dec 75	Major Richard A. Kishimoto, MSC Aerobiology Division	Interaction between guinea pig peritoneal macrophages and <u>Coxiella burnetii</u> <u>in vitro</u> .
23 Jan 76	Dr. Robert W. Wannemacher, Jr., Ph.D. Physical Sciences Division	Endogenous amino acid utilization in infected hosts.
	Dr. Harold A. Neufeld, Ph.D. Physical Sciences Division	The inhibition of fasting-induced ketosis by bacterial infection and inflammation.
	Lt Colonel Mitchell V. Kaminski, Jr., MC Physical Sciences Division	Uninhibited ketosis in non-septic stress: Hypothesized controls and proposed therapeutic manipulation by micronutrient infusions.
	Dr. Carol N. Mapes, Ph.D. Physical Sciences Division	Possible interrelationships of prostaglandins and leukocytic endogenous mediators.
	Lt Colonel Philip Z. Sobocinski, MSC Physical Sciences Division	Some observations on the efficacy of zinc treatment in preventing endotoxin-induced mortality.
20 Feb 76	Lt Colonel William E. Houston, MSC Animal Assessment Division	Effects of PIC-L on the primary immune response to inactivated VEE vaccine.
	Captain Myrl L. Sammons, VC Animal Assessment Division	Dose response study to modified poly I-C in rhesus monkeys.
	Major Edward L. Stephen, VC Animal Assessment Division	Aerosol therapy of influenza infections of mice and primates with Rimantadine, Ribavirin and related compounds.
	Major Donald G. Harrington, VC Animal Assessment Division	Response of monkeys to intranasal challenge with Japanese encephalitis virus.
	Dr. C. T. Liu, Ph.D. Animal Assessment Division	A new look on an old problem: SEB toxicity in rhesus monkeys.

<u>DATE</u>	<u>LECTURER</u>	<u>TITLE OF PRESENTATION</u>
19 Mar 76	Dr. Stephen H. Leppla, Ph.D. Pathology Division	<u>Pseudomonas aeruginosa</u> exotoxin: Chemical properties and role of pathogenesis.
	Dr. John L. Middlebrook, Ph.D. Pathology Division	Studies on the mechanism of action of <u>Pseudomonas aeruginosa</u> exotoxin.
	Captain David A. Wing, MSC Pathology Division	Protein turnover studies as a tool in studying disseminated intravascular coagulation.
	Major Arthur O. Anderson, MC Pathology Division	The response of the regional lymph node to adjuvant and adjuvant-like substances.
23 Apr 76	Dr. Joseph V. Jemski, Ph.D. Aerobiology Division	Respiratory infection with <u>Mycoplasma pneumoniae</u> in the hamster. I. Aerosol studies.
	Dr. John D. White, Ph.D. Pathology Division	Respiratory infection with <u>Mycoplasma pneumoniae</u> in the hamster. II. SEM studies.
	Dr. Richard F. Berendt, Ph.D. Aerobiology Division	Studies on the use of aerosols of antiviral drugs for the therapy of experimental influenza in squirrel monkeys.
	Major John B. Arensman, VC Animal Assessment Division	Effects of infection and aerosols of antiviral drugs on selected respiratory parameters in the influenza mouse model.
21 May 76	Major Robert L. Hickman, VC Animal Resources Division	Laboratory animals used at USAMRIID - their attributes and limitations.
	Captain Bobby R. Collins, VC Animal Resources Division	Humane care of laboratory animals in biomedical research - legal and moral responsibilities.
	Captain Gregory B. Heisey, VC Animal Resources Division	The cotton rat (<u>Sigmodon hispidus</u>) - a model for VEE infection.
	Mr. William C. Patrick, III Plans and Programs Officer	Peer review and USAMRIID productiv- ity.

APPENDIX D

U.S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
FORMAL PRESENTATIONS AND BRIEFINGS BY STAFF

<u>Date and Group or Individual</u>	<u>Participating Staff Member(s)</u>	<u>Title</u>
14-16 Jul 75 International Symposium on Arboviral Infection of Public Health Importance Communicable Disease Center Atlanta, Georgia	Lt Colonel Gerald A. Eddy, VC	Pathogenesis of Machupo virus infection in primates.
		Protection of monkeys against Machupo virus by the passive administration of human Bolivian hemorrhagic fever immune globulin.
28 Jul 75 Seminar, Department of Medicine University of Virginia School of Medicine Charlottesville, Virginia	Dr. Harold A. Neufeld, Ph.D.	Effect of bacterial infection on ketone body concentrations in rat liver and blood.
3-9 Aug 75 Symposium on Nutrition and Infection Dr. Robert W. Wannemacher, Jr., Ph.D. at the Xth International Congress of Nutrition Kyoto, Japan		Effects of infection on host metabolism.
12 Aug 75 Doris T. Donner, Frederick C. Kass, Office of Congressman Downey (NY) Iris Portney, Office of Congresswoman Schroeder (CO) Major George Jacumski, Congressional Liaison, Office of Secretary of the Army, Washington, D.C.	Colonel Joseph F. Metzger, MC Lt Colonel David D. Dryden, MSC	Briefing on USAMRIID mission and aspects of program on topics desired by Congressman Downey.

<u>Date and Group or Individual</u>	<u>Participating Staff Member(s)</u>	<u>Title</u>
19-20 Aug 75 Annual Command Visit	Colonel Joseph F. Metzger, MC Colonel F. C. Cadigan, Jr., MC, Captain C. J. Schaeffer, MC, Lt Colonel R. O. Spertzel, VC, Lt Colonel F. L. Coddington, MSC, Lt Colonel H. P. Stoltz, VC, Major I. Ayala, MSC, Captain R.V.N. Ginn, MSC, Captain J. N. Shkittka, JAGC, and Major P. J. Leach, MSC, all of U.S. Army Medical Research and Development Command, Washington, D.C.	Annual orientation briefing of overall mission and operation of USAMRIID; and entire staff tour of facilities.
26-29 Aug 75	Symposium on Antivirals with Clinical Major Jerry S. Walker, VC Potential, sponsored by NIAID and Infectious Diseases Society Stanford University School of Medicine Stanford, California	The use of small-particle aerosols of antiviral compounds for the treatment of type A influenza pneumonia in animal models.
11-12 Sep 75	Mr. Claude Chabassol French Ministry of Defense Section d'Etudes de Biologie et de Chimie Boite Postale n 3, 91710 Vert le PETIT, France	Colonel Harry G. Dangerfield, MC Dr. William R. Beisel, M.D. and staff
15-19 Sep 75	Pathology of Laboratory Animals Education Course Armed Forces Institute of Pathology Washington, D.C.	Participant in the faculty of the course; and presenter of lecture on respiratory diseases of nonhuman primates.

<u>Date and Group or Individual</u>	<u>Participating Staff Member(s)</u>	<u>Title</u>
24-26 Sep 75 15th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy Washington, D.C.	Dr. Richard F. Berendt, Ph.D.	Fate of aerosols of Kanamycin in normal and respiratory <u>Klebsiella pneumoniae</u> -infected rats.
	Major Cyril M. Hetsko, MC	Experimental infection of hamsters with aerosols of <u>Mycoplasma pneumoniae</u> .
	Major Edward L. Stephen, VC	Therapeutic effects of Ribavirin given by the intraperitoneal and aerosol routes in influenza-infected mice.
	Captain Peter J. Felsburg, MSC	The active-E rosette test: A sensitive <u>in vitro</u> assay for human delayed type hypersensitivity to microbial antigens.
	Mr. Stephen J. Little	The active-E rosette test: Correlation with delayed cutaneous hypersensitivity.
		Effectiveness of antibiotics for therapy of murine tularemia.
5-8 Oct 75 The Second International Workshop on Basic Properties and Clinical Applications of Transfer Factor held at USAMRIID, Fort Detrick, Frederick, Maryland	Major Michael S. Ascher, MC	In <u>vitro</u> properties of leukocyte dialysates containing transfer factor: Micro method and recent findings. Chromatography of transfer factor and assay of fractions <u>in vitro</u> .
5-10 Oct 75 American Physiological Society Meeting San Francisco, California	Dr. Ching-Tong Liu, Ph.D.	Effect of staphylococcal enterotoxin B on body fluid compartments in conscious rhesus monkeys.

<u>Date and Group or Individual</u>	<u>Participating Staff Member(s)</u>	<u>Title</u>
9 Oct 75 Major Michael Belkin, M.D. Israeli Defense Forces Hadassah Medical Center Jerusalem	Dr. William R. Beisel, M.D. Colonel Harry G. Dangerfield, MC	General briefing on mission of USAMRIID and its facilities.
23-24 Oct 75 Symposium on Basic Biology of Rickettsiae Walter Reed Army Institute of Research Washington, D.C.	Dr. Ralph F. Wachter, Ph.D.	Changes in buoyant density relationships of two cell types of <u>Coxiella burnetii</u> Phase I.
29 Oct 75 Frederick County Association of Science Teachers Frederick, Maryland	Dr. Harold A. Neufeld, Ph.D.	Effect of infection on ketone body concentrations in the rat liver.
10-14 Nov 75 Meeting of the American Society of Tropical Medicine and Hygiene New Orleans, Louisiana	Lt Colonel Gerald A. Eddy, VC	Cross protection of Machupo virus in- fected monkeys by viruses of the Tacaribe complex.
	Lt Colonel Robert Edelman, MC	The attenuated VEE vaccines: Their antigenic potency in man.
	Major Carl E. Pedersen, Jr., MSC	Studies on antigenic components of selected alphaviruses: Immunogenicity of envelope components.
11-14 Nov 75 15th Annual Meeting of the American Society for Cell Biology San Juan, Puerto Rico	Captain Michael C. Powanda, MSC	Systemic metabolic consequences of phagocytosis.

<u>Date and Group or Individual</u>	<u>Participating Staff Member(s)</u>	<u>Title</u>
16-21 Nov 75 Fourth Asian and Oceanian Congress of Neurology Bangkok, Thailand	Lt Colonel Robert Edelman, MC Captain Leo A. Andron, II, MSC	Persistence of JEV IgM antibody and clinical recovery after Japanese encephalitis.
18 Nov 75 Clinical Immunology Course The Johns Hopkins University School of Medicine Baltimore, Maryland	Major Michael S. Ascher, MC Captain Leo A. Andron, II, MSC	Transfer factor.
18 Nov 75 Medical Aspects of Advanced Warfare Course U.S. Air Force School of Aerospace Medicine Brooks AFB, Texas	Colonel Harry G. Dangerfield, MC	Medical defense against biological agents.
20-21 Nov 75 1975 USAMRIID Planning Session with the Ad Hoc Study Group for Special Infectious Disease Problems Fort Detrick, Maryland	Colonel Joseph F. Metzger, MC Brigadier General Kenneth R. Dirks, MC Dr. William R. Beisel, M.D.	Greetings and discussion of agenda. Introductory remarks. The current status of USAMRIID and its research activities.
	Lt Colonel Robert Edelman, MC	Subpopulation of actively rosetting T-lymphocytes as an index of CMI in man.
	Dr. Carol A. Mapes, Ph.D.	Multiple leukocytic factors that induce reactions characteristic of the granulomatous inflammatory response.
	Lt Colonel Gerald A. Eddy, VC	Arenavirus studies at USAMRIID.

Date and Group or Individual

Participating Staff Member(s)

Title

20-21 Nov 75
USAMRIID Planning Session
(continued)

The African Green Monkey as an alternative primate host for studying Machupo virus infections:

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|----------------------------------|---------------------------------------------------------------------------------------------------------------------|
| Captain Franklin S. Wagner, VC | Clinical aspects. |
| Major Charles G. McLeod, Jr., VC | Pathogenic studies of Bolivian hemorrhagic fever. |
| Major Carl E. Pedersen, Jr., MSC | Overview of Rickettsiology Division research. |
| Dr. Richard H. Kenyon, Ph.D. | Immunological aspects of spotted fever vaccines. |
| Major Charles N. Oster, MC | Diagnosis of Rocky Mountain spotted fever using radioimmunoassay techniques. |
| Major Michael S. Ascher, MC | Specific <u>in vitro</u> lymphocyte transformation to Rocky Mountain spotted fever rickettsial antigen. |
| Dr. Ralph F. Wachter, Ph.D. | Immunological potential of the soluble antigen of <u>Coxiella burnetii</u> . |
| Major Richard A. Kishimoto, MSC | Interaction between guinea pig peritoneal macrophages and <u>Coxiella burnetii</u> in vitro. |
| Major Edward L. Stephen, VC | Effects of poly (ICLC) on yellow fever, Machupo, and Venezuelan equine encephalomyelitis virus diseases in monkeys. |
| Mr. Ralph W. Kuehne, M.S. | Mouse models for evaluating potential antiviral compounds: A new "indirect" evaluation model. |

<u>Date and Group or Individual</u>	<u>Participating Staff Member(s)</u>	<u>Title</u>
20-21 Nov 75 USAMRIID Planning Session (continued)	Major Jerry S. Walker, VC	The use of small-particle aerosols of antiviral compounds for the treatment of type A influenza pneumonia in animal models.
	Dr. Richard F. Berendt, Ph.D.	Pharmacokinetic aspects of aerosols of Kanamycin in normal and respiratory <u>Klebsiella pneumoniae</u> -infected rats.
	Dr. Leonard Spero, Ph.D.	Overview of toxin research.
	Dr. Stephen H. Leppla, Ph.D.	<u>Pseudomonas exotoxin</u> --Properties and role in pathogenesis.
	Dr. John L. Middlebrook, Ph.D.	The response of mammalian cells to the exotoxins of <u>Corynebacterium diphtheriae</u> and <u>Pseudomonas aeruginosa</u> : Differential cytotoxicity.
	Ms. Anna D. Johnson	Staphylococcal exfoliative toxin.
5 Dec 75	Dr. William R. Beisel, M.D.	Report on the activities of the sub-committee on interactions of nutrition and infections.
	Assembly of Life Sciences Food and Nutrition Board Symposium National Research Council Washington, D.C.	
13 Dec 75	Annual Meeting of Veterinary Medical Major Edward L. Stephen, VC Section Association of Military Surgeons of the U.S. Washington, D.C.	Advances in viral chemotherapy.

<u>Date and Group or Individual</u>	<u>Participating Staff Member(s)</u>	<u>Title</u>
16 Dec 75 Dr. R. Fontanges Centre de Recherche du Service de Santé des Armées Lyon, France	Dr. William R. Beisel, M.D. and staff	Briefings of USAMRIID research programs of mutual interest. Visit approved through Department of Defense channels.
16 Dec 75 Mr. William Foyars Dr. Dorland Davis Members, House Appropriations Committee, Washington, D.C.	Colonel Joseph F. Metzger, MC Dr. William R. Beisel, M.D.	Briefing on USAMRIID mission and research program.
8 Jan 76 1976 Meeting of the U.S. Army Medical Research and Development Advisory Panel, held at USAMRIID, Fort Detrick, Maryland	Colonel Joseph F. Metzger, MC Brigadier General Kenneth R. Dirks, MC	Opening remarks.
	Lt Colonel Gerald A. Eddy, VC	USAMRIID studies with Machupo virus and Bolivian hemorrhagic fever.
	Lt Colonel Robert Edelman, MC	Attenuated VEE vaccine: Review of its effectiveness and long-term consequences.
	Lt Colonel William E. Houston, MSC	Research studies concerning adjuvants for use with weakly antigenic vaccines.
15 Jan 76 Dr. H. L. Ley, Jr., Chairman, LTG A. W. Betts, USA (Ret), Dr. R.A. Dr. William R. Beisel, M.D. Beaudet, Dr. C.J.D. Zarafonetis, Dr. J. L. Steinfield, all members of an Ad Hoc Group on Review of Bio- logical and Toxin Samples within Department of the Army.	Colonel Joseph F. Metzger, MC and staff	Review of inventory of biological and toxin samples on hand at USAMRIID.
16 Jan 76 Medical Aspects of Advanced Warfare Course, US Air Force Sch of Aero- space Medicine, Brooks AFB, Texas	Colonel Harry G. Dangerfield, MC	Medical defense against biological agents.

<u>Date and Group or Individual</u>	<u>Participating Staff Member(s)</u>	<u>Title</u>
22-24 Jan 76 Meeting of the Southern Section of the American Federation for Clinical Research New Orleans, Louisiana	Major Douglas P. Fine, MC	Role of complement in experimental Rocky Mountain spotted fever.
26 Jan 76 CAPT Cornelius J. Breden, USAF, LT Richard J. Keefe, USN 3428th Technical Training Squadron Offutt, AFB, Nebraska	Dr. William R. Beisel, M.D.	Briefing on USAMRIID research program and tour of facilities.
2-5 Feb 76 3d Conference on Antiviral Substances New York Academy of Sciences New York, New York	Major Edward L. Stephen, VC	Aerosol therapy of influenza infections of mice and primates with Rimantadine, Ribavirin and related compounds.
5 Mar 76 Department of Biochemistry University of Rochester Medical School, Rochester, New York	Dr. Harold A. Neufeld, Ph.D.	The effects of septic and nonseptic stress on ketosis in the rat.
11 Mar 76 Dr. John T. Bartlett Microbiological Research Establishment Porton Down, England	Dr. William R. Beisel, M.D. and staff members	Briefings and discussions of current USAMRIID programs of mutual interest. Visit approved through Department of Defense channels.
17 Mar 76 Department of Pediatrics Seminar University of Pennsylvania Philadelphia, Pennsylvania	Dr. William R. Beisel, M.D.	Complexities of the metabolic response to infection.

<u>Date and Group or Individual</u>	<u>Participating Staff Member(s)</u>	<u>Title</u>
11-13 Mar 76 The Medical College of Wisconsin Milwaukee County General Hospital Milwaukee, Wisconsin	Major H. Bradford Hawley, MC	Pneumococcal sepsis and disseminated intravascular coagulation.
15 Mar 76 Seminar, Department of Basic and Clinical Immunology and Microbiology Medical University of S. Carolina Charleston, South Carolina	Major Michael S. Ascher, MC	In vitro studies of transfer factor.
23-27 Mar 76 International Academy of Pathology Conference Boston, Massachusetts	Captain William C. Hall, VC	The effects of acute vitamin A deficiency on the immune response.
24 Mar 76 Veterinary College Seminar Cornell University Ithaca, New York	Dr. William R. Beisel, M.D.	Metabolic responses to infection in animals and man.
25 Mar 76 Infection and Nutrition Post-graduate Course Departments of Medicine and Surgery Columbia University New York, New York	Dr. William R. Beisel, M.D.	The metabolic response to infection.
9 Apr 76 LTC Dr. A. Kvill Deputy Surgeon General Israeli Defense Forces Israel	Colonel Joseph F. Metzger, MC Dr. William R. Beisel, M.D.	Briefing on USAMRIID mission and research program; tour of facilities. Visit authorized through Department of Defense.

<u>Date and Group or Individual</u>	<u>Participating Staff Member(s)</u>	<u>Title</u>
12-16 Apr 76 Annual Meeting, Federation of American Societies for Experimental Biology Anaheim, California	Lt Colonel Phillip Z. Sobocinski, MSC Major Arthur O. Anderson, MC Major Michael S. Ascher, MC	Effect of zinc pretreatment on endotoxin-induced mortality and hyperaminoacidemia in rats. The mechanism of lymphocyte homing. Transfer factor <u>in vitro</u> : Nonspecificity of leukocyte lysates that enhance lymphocyte proliferation to antigen.
	Captain Leo A. Andron, II, MSC	Transfer factor <u>in vitro</u> : Chromatography of components that enhance antigen induced lymphocyte transformation.
	Captain Hardy M. Howell, MSC	Augmentation of phytomitogen induced lymphocyte transformation.
	Dr. Richard H. Kenyon, Ph.D.	Lymphocyte transformation to rickettsial antigen in Rocky Mountain spotted fever.
	Dr. Robert W. Wannemacher, Jr., Ph.D.	Hepatic gluconeogenic capacity and rate during pneumococcal infection in rats.
23 Apr 76 Briefing - BW Defense Public Health Service Section #1	Colonel Harry G. Dangerfield, MC	Medical defense against biological agents.
24 Apr 76 Maryland-Washington, D.C. Branch Meeting of the American Society for Microbiology Fort Detrick, Maryland	Dr. William R. Beisel, M.D. Dr. Bruno J. Luscri, Ph.D.	Welcome and opening remarks. Interferon sensitivity and induction with selected arenaviruses.

<u>Date and Group or Individual</u>	<u>Participating Staff Member(s)</u>	<u>Title</u>
24 Apr 76 Maryland-Washington, D.C. Branch Meeting of the American Society for Microbiology (continued)	Major Richard A. Kishimoto, MSC	An electron microscopic study on the interaction between normal guinea pig peritoneal macrophages and <u>Coxiella burnetii</u> .
28-30 Apr 76 Conference on Biomedical Role of Trace Elements in Aging Eckerd College St. Petersburg, Florida	Dr. Robert W. Wannemacher, Jr., Ph.D.	Trace element changes during infection at various ages.
1-2 May 76 Annual Meeting, American Society for Clinical Nutrition Atlantic City, New Jersey	Lt Colonel Mitchell V. Kaminski, MC	Postoperative amino acid infusion, nitrogen sparing and beneficial ketosis-A glucagon and growth hormone effect.
2-7 May 76 76th Annual Meeting, American Society for Microbiology Atlantic City, New Jersey	Major Richard A. Kishimoto, MSC	An electron microscopic study on the interaction between normal guinea pig peritoneal macrophages and <u>Coxiella burnetii</u> .
	Captain Joseph D. Gangemi, MSC	Biochemical analysis of the polypeptide composition of several arenaviruses.
	Captain Myrl L. Sammons, VC	Serum interferon response in rhesus monkeys to modified polyriboinosinic-polyribocytidylic acid complex.
	Dr. Bruno J. Luscri, Ph.D.	Interferon sensitivity and induction with selected arenaviruses.
	Mr. Ralph W. Ruehne	An indirect mouse model for evaluating potential antiviral compounds.

<u>Date and Group or Individual</u>	<u>Participating Staff Member(s)</u>	<u>Title</u>
11-13 May 76 Workshop on Impact of Infection on Nutritional Status of the Host Subcommittee on Interactions of Nutrition and Infections of the National Research Council/National Academy of Sciences Warrenton, Virginia	Lt Colonel Mitchell V. Kaminetz, Jr., MC Major Michael C. Powanda, MSC	Specific metabolic effects imposed by <u>Streptococcus pneumoniae</u> upon the response to femoral fracture in the rat. Changes in body balance nitrogen and other key nutrients: Description and underlying mechanisms.
	Dr. Peter G. Canonico, Ph.D.	The effects of pneumococcal infection on rat liver microsomal enzymes and lipogenesis.
	Dr. Robert W. Wannemacher, Jr., Ph.D.	Total body protein catabolism in starved and infected rats.
	Dr. Harold A. Neufeld, Ph.D.	Key role of various individual amino acids in host response to infection.
	Dr. William R. Beisel, M.D.	Effect of inflammatory and noninflammatory stress on ketone bodies and free fatty acids in rats.
		Magnitude of the host nutritional responses to infection.
		Impact of infection on nutritional status of the host. Definition of the problem and objectives of the Workshop.
		Summary of the Workshop: Concluding remarks.

<u>Date and Group or Individual</u>	<u>Participating Staff Member(s)</u>	<u>Title</u>
17 May 76 Seminar, Section of Infectious Diseases Department of Medicine Medical College of Wisconsin Milwaukee, Wisconsin	Major Michael S. Ascher, MC	Transfer factor.
20 May 76 Annual Meeting, The Maryland Dietetic Association Beltsville, Maryland	Lt Colonel Mitchell V. Kaminski, Jr., MC	Enteral and parenteral hyperalimentation- Indications and practice for various clinical groups.
20 May 76 Seminar, The Johns Hopkins University Medical School Baltimore, Maryland	Lt Colonel Gerald A. Eddy, VC	Bolivian hemorrhagic fever studies in primates.
27-28 May 76 Dr. Ake H. A. Bovalius, Dr. Nils Gustaf F. Nilsson National Defense Research Institute Sweden	Colonel Joseph F. Metzger, MC Dr. William R. Beisel, M.D. and staff members	Briefing on USARIID mission and research program; tour of facilities. Visit authorized through Department of Defense.
6-10 Jun 76 American Society of Biological Chemists Meeting San Francisco, California	Dr. John L. Middlebrook, Ph.D.	Response of mammalian cells to the exotoxins of <u>Corynebacterium diphtheriae</u> and <u>Pseudomonas aeruginosa</u> : Differential cytotoxicity.
	Dr. Harold A. Neufeld, Ph.D.	The effect of inflammatory and non-inflammatory stress on kerosis in the rat.
	Dr. Leonard Spero, Ph.D.	Limited tryptic hydrolysis of staphylococcal enterotoxin C.
	Dr. Robert W. Wannemacher, Jr.; Ph.D.	Total body protein catabolism in starved and infected rats.

<u>Date and Group or Individual</u>	<u>Participating Staff Member(s)</u>	<u>Title</u>
17 Jun 76 Seminar, Staff Members of the Department of Microbiology Indiana University School of Medicine Indianapolis, Indiana	Dr. William R. Beisel, M.D.	Unique phagocytic and hepatic cell interactions as a general form of host response to invading microorganisms.
Presentation to students of the Indiana University School of Medicine Indianapolis, Indiana	Dr. Beisel	Leukocyte mediators.
21 Jun 76 Meeting on Testing of Influenza Vaccines, sponsored by National Institute of Allergy and Infectious Diseases, Bureau of Biologics, Communicable Disease Center, and the Department of Defense, held at National Institutes of Health Bethesda, Maryland	Lt Colonel Robert Edelman, MC	Results of tests of bivalent A vaccine and of the concurrent use of bivalent A and monovalent B vaccines.
22-25 Jun 76 1976 Army Science Conference U.S. Military Academy West Point, New York	Dr. Carol A. Mapes, Ph.D.	Multiple leukocytic factors that induce reactions characteristic of the inflammatory responses.
	Major Edward L. Stephen, VC	First successful use of a chemical compound for the prophylaxis and treatment of a lethal, systemic, viral infection common to man and subhuman primates.
23-25 Jun 76 58th Annual Meeting of the Endocrine Society San Francisco, California	Captain David T. George, MSC	Alterations in plasma insulin, glucagon, glucose, hepatic cyclic AMP and liver glycogen by a leukocyte derived factor(s). 471

APPENDIX E
PUBLICATIONS OF U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
FISCAL YEAR 1976

1. Altenbernd, R. A. 1975. Derivation of high enterotoxin B-producing mutants of Staphylococcus aureus from the parent strain. *Appl. Microbiol.* 30:271-275.
2. Altenbernd R. A. 1976. Enterotoxin B formation by fermentation mutants of Staphylococcus aureus. *Can. J. Microbiol.* 22:182-188.
3. Anderson, A. O., and N. D. Anderson. 1976. Lymphocyte emigration from high endothelial venules in rat lymph nodes. *Immunology* 31:731-748.
4. Anderson, N. D., A. O. Anderson, and R. G. Wyllie. 1976. Specialized structure and metabolic activities of high endothelial venules in rat lymphatic tissues. *Immunology* 31:455-473.
5. Andron, II, L. A., and H. T. Eigelsbach. 1975. Biochemical and immunological properties of ribonucleic acid-rich extracts from Francisella tularensis. *Infect. Immun.* 12:137-142.
6. Andron, L. A., and M. S. Ascher. 1976. Chromatography of transfer factor and assay of fractions *in vitro*. p. 291-297. Discussion, p. 298-300. *In Transfer Factor* (ed. M. S. Ascher, A. A. Gottlieb, and C. H. Kirkpatrick). Academic Press, New York.
7. Ascher, M. S., and L. A. Andron. 1976. *In vitro* properties of leukocyte dialysates containing transfer factor: micro method and recent findings. p. 3-9. Discussion, p. 10-12. *In Transfer Factor* (ed. M. S. Ascher, A. A. Gottlieb, and C. H. Kirkpatrick). Academic Press, New York.
8. Ascher, M. S., A. A. Gottlieb, and C. H. Kirkpatrick (eds). 1976. *Transfer Factor*. Academic Press, New York. 757 pp.
9. Ayala, E., and P. G. Canonico. 1975. Aminoisobutyric acid transport in primary cultures of normal adult rat hepatocytes. *Proc. Soc. Exp. Biol. Med.* 149:1019-1022.
10. Bailey, P. T., F. B. Abeles, E. C. Hauer, and C. A. Mapes. 1976. Intracerebroventricular administration of leukocytic endogenous mediators (LEM) in the rat. *Proc. Soc. Exp. Biol. Med.* 152: in press.
11. Bartelloni, P. J., and R. B. Tesh. 1976. Clinical and serologic responses of volunteers infected with Phlebotomus fever virus (Sicilian type). *Am. J. Trop. Med. Hyg.* 25:456-462.
12. Beisel, W. R. 1975. The effects of malnutrition on immunological responses of the host: the key role of scarce amino acids. *In Western Hemisphere Nutrition Congress IV* (ed. P. L. White and N. Selvey). p. 313-318. Publishing Sciences Group, Inc. Acton, MA.

RECORDED PAGE BLANK-NOT FILMED

13. Beisel, W. R. 1976. Enterotoxin-mediated disease, Chapter 1, p.1-66. In *Trace Substances and Health* (ed. P. M. Newberne). Marcel Dekker, New York.
14. Beisel, W. R. 1976. The influence of infection or injury on nutritional requirements during adolescence. p. 257-278. In *Nutrient Requirements in Adolescence* (ed. J. I. McKigney and H. N. Munro). MIT Press, Boston.
15. Beisel, W. R. 1976. Trace elements in infectious processes. *Med. Clin. N. Am.* 60:831-849.
16. Beisel, W. R., R. S. Pekarek, and R. W. Wannemacher, Jr. 1976. Homeostatic mechanisms affecting plasma zinc levels in acute stress. p. 87-106. In *Trace Elements in Human Health and Disease*, Vol. 1 (ed. A. S. Prasad). Academic Press, New York.
17. Beisel, W. R. 1976. Nonspecific host defensive factors. In *Malnutrition and the Immune Response* (ed. R. M. Suskind). The Raven Press, New York, in press.
18. Beisel, W. R. 1976. Malnutrition as a consequence of stress. In *Malnutrition and the Immune Response* (ed. R. M. Suskind). The Raven Press, New York, in press.
19. Beisel, W. R., G. L. Cockerell, and W. A. Janssen. 1976. Nutritional effects on the responsiveness of plasma acute-phase reactant glycoproteins. In *Immune Status in Malnutrition* (ed. R. Suskind). The Raven Press, New York, in press.
20. Beisel, W. R. 1976. Impact of infection on nutritional status of the host. *Am. J. Clin. Nutr.*, in press.
21. Beisel, W. R. 1976. Magnitude of the host nutritional responses to infection. *Am. J. Clin. Nutr.*, in press.
22. Beisel, W. R. 1976. Effect of nutrition on nutritional needs. In *CRC Handbook of Nutrition and Foods* (ed. M. Rechcigl). CRC Press, Cleveland, OH, in press.
23. Berendt, R. F., G. G. Long, and J. S. Walker. 1975. Treatment of respiratory Klebsiella pneumoniae infection in mice with aerosols of kanamycin. *Antimicrob. Agents Chemother.* 8:585-590.
24. Berendt, R. F., G. G. Long, and J. S. Walker. 1975. Influenza alone and in sequence with pneumonia due to Streptococcus pneumoniae in the squirrel monkey. *J. Infect. Dis.* 132:689-693.
25. Berendt, R. F., and J. S. Walker. 1976. Distribution of kanamycin in rat tissues after aerosol or intramuscular treatment. *Antimicrob. Agents Chemother.* 10: in press.
26. Bostian, K. A., B. S. Blackburn, R. W. Wannemacher, Jr., V. G. McGann, W. R. Beisel, and H. L. DuPont. 1976. Sequential changes in the concentration of specific serum proteins during typhoid fever infection in man. *J. Lab. Clin. Med.* 87:577-585.

27. Buhles, W. C., D. L. Huxsoll, G. Ruch, R. H. Kenyon, and B. L. Elisberg. 1975. Evaluation of primary blood monocyte and bone marrow cell culture for the isolation of Rickettsia rickettsii. *Infect. Immun.* 12:1457-1463.
28. Burke, D. S., and R. Edelman. 1976. The use of attenuated Venezuelan equine encephalomyelitis (VEE) vaccines in man: a review. *Arthropod-Borne Virus Information Exchange* 30:143-144.
29. Canonico, P. G., M. C. Powanda, G. L. Cockerell, and J. B. Moe. 1975. Relationship of serum β -glucuronidase and lysozyme to pathogenesis of tularemia in immune and nonimmune rats. *Infect. Immun.* 12:42-47.
30. Canonico, P. G., J. D. White, and M. C. Powanda. 1975. Peroxisome depletion in rat liver during pneumococcal sepsis. *Lab. Invest.* 33: 147-150.
31. Canonico, P. G., E. Ayala, W. L. Rill, and J. S. Little. 1976. The effects of pneumococcal infection on rat liver microsomal enzymes and lipogenesis. *Am. J. Clin. Nutr.*, in press.
32. Cole, Jr., F. E., C. E. Pedersen, Jr., D. M. Robinson, and G. A. Eddy. 1976. Improved method for production of attenuated Venezuelan equine encephalomyelitis (TC-83 strain) vaccine. *J. Clin. Microbiol.* 3:460-462.
33. Curnow, R. T., E. J. Rayfield, D. T. George, T. V. Zenser, and F. R. DeRubertis. 1976. Altered hepatic glycogen metabolism and glucoregulatory hormones during sepsis. *Am. J. Physiol.* 230:1296-1301.
34. DeRubertis, F. R., and T. Zenser. 1976. Activation of murine lymphocytes by cyclic guanosine 3',5'-monophosphate: specificity and role in mitogen action. *Biochim. Biophys. Acta* 428:91-102.
35. Eddy, G. A., S. K. Scott, F. S. Wagner, and O. M. Brand. 1975. Pathogenesis of Machupo virus infection in primates. *Bull. WHO* 52:517-521.
36. Eddy, G. A., F. S. Wagner, S. K. Scott, and B. J. Mahlandt. 1975. Protection of monkeys against Machupo virus by the passive administration of Bolivian haemorrhagic fever immunoglobulin (human origin). *Bull. WHO* 52: 723-727.
37. Edelman, R. 1976. Cell-mediated immune response in protein-calorie malnutrition--A review. In *Malnutrition and the Immune Response* (ed. R. M. Suskind), Raven Press, New York, in press.
38. Edelman, R., P. Kulapongs, R. Suskind, and R. E. Olson. 1976. Defective leukocyte mobilization in children with kwashiorkor. In *Malnutrition and the Immune Response* (ed. R. M. Suskind), Raven Press, New York, in press.
39. Edelman, R. 1976. Clinical trial of inactivated, TC-83 Venezuelan equine encephalomyelitis vaccine. *Arthropod-Borne Virus Information Exchange* 31:29-30.

40. Eigelsbach, H. T., D. H. Hunter, W. A. Janssen, H. G. Dangerfield, and S. G. Rabinowitz. 1975. Murine model for study of cell-mediated immunity: protection against death from fully virulent Francisella tularensis infection. *Infect. Immun.* 12:999-1005.
41. Elwell, M. R., C. T. Liu, R. O. Spertzel, and W. R. Beisel. 1975. Mechanisms of oral staphylococcal enterotoxin B-induced emesis in the monkey. *Proc. Soc. Exp. Biol. Med.* 148:424-427.
42. Elwell, M. R., M. L. Sammons, C. T. Liu, and W. R. Beisel. 1975. Changes in blood pH in rats after infection with Streptococcus pneumoniae. *Infect. Immun.* 11:724-726.
43. Faulkner, R. T., D. G. Harrington, M. L. Sammons, D. E. Hilmas, and L. Sammons. 1976. A case of esophageal foreign body with mediastinal abscess formation in a dog. *J. Am. Anim. Hosp. Ass.* 12:70-76.
44. Faulkner, R. T., W. P. Czajkowski, D. G. Harrington, F. E. Chapple, and R. L. Hickman. 1976. Internal fixation of humeral fractures in rhesus monkeys (Macaca mulatta). *Vet. Med. Small Anim. Clin.* 71:643-647.
45. Faulkner, R. T., W. P. Czajkowski, E. J. Rayfield, and R. L. Hickman. 1976. Technique for portal catheterization in rhesus monkeys (Macaca mulatta). *Am. J. Vet. Res.* 37:473-476.
46. Felsburg, P. J., R. Edelman, and R. H. Gilman. 1976. The active E rosette test: correlation with delayed cutaneous hypersensitivity. *J. Immunol.* 116:1110-1114.
47. Fine, D. P. 1975. Pneumococcal type-associated variability in alternate complement pathway activation. *Infect. Immun.* 12:722-778.
48. Harrington, D. G., M. R. Elwell, and D. E. Hilmas. 1976. Observations on the response of monkeys to intranasal challenge with Japanese encephalitis virus. *Arthropod-Borne Virus Information Exchange* 30:141-142.
49. Hilmas, D. E., and R. O. Spertzel. 1975. Response of sublethally irradiated monkeys to a replicating viral antigen. *Infect. Immun.* 12:592-601.
50. Houston, W. E., C. L. Crabbs, R. J. Kremer, and J. W. Springer. 1976. Adjuvant effects of diethylaminoethyl-dextran. *Infect. Immun.* 13: 1559-1562.
51. Houston, W. E., C. L. Crabbs, E. L. Stephen, and H. B. Levy. 1976. Modified polyriboinosinic-polyribocytidylic acid, and immunological adjuvant. *Infect. Immun.* 14:318-319.
52. Hughes, F., and C. E. Pedersen, Jr. 1975. Paramagnetic spin label interactions with the envelope of a group A arbovirus. Lipid organization. *Biochim. Biophys. Acta* 394:102-110.

53. Jahrling, P. B. 1975. Interference between virulent and vaccine strains of Venezuelan encephalitis virus in mixed infections of hamsters. *J. Gen. Virol.* 28:1-8.
54. Jahrling, P. B., and L. Gorelkin. 1975. Selective clearance of a benign clone of Venezuelan equine encephalitis virus from hamster plasma by hepatic reticuloendothelial cells. *J. Infect. Dis.* 132:667-676.
55. Jahrling, P. B. 1976. Chromatographic separation of Venezuelan and Western encephalitis virus subtypes. *Arthropod-Borne Virus Information Exchange* 30:137-140.
56. Jahrling, P. B. 1976. Virulence heterogeneity of a predominantly avirulent Western equine encephalitis virus population. *J. Gen. Virol.* 121-128.
57. Jahrling, P. B., E. Navarro, and W. F. Scherer. 1976. Interferon induction and sensitivity as correlates to virulence of Venezuelan encephalitis virus for hamsters. *Arch. Virol.* 51:23-35.
58. Jemski, J. V., and J. S. Walker. 1976. Aerosol vaccination of mice with a live, temperature-sensitive recombinant influenza virus. *Infect. Immun.* 13:818-824.
59. Johnson, A. D., J. F. Metzger, and L. Spero. 1975. Production, purification, and chemical characterization of Staphylococcus aureus exfoliative toxin. *Infect. Immun.* 12:1206-1210.
60. Johnson, J. W., G. A. Eddy, and C. E. Pedersen, Jr. 1976. Biological properties of the M-44 strain of Coxiella burnetii. *J. Infect. Dis.* 133:334-338.
61. Kaminski, Jr., M. V., H. A. Neufeld, R. W. Wannemacher, Jr., M. L. Armstrong, and L. Strobel. 1976. Specific metabolic effects imposed by S. pneumoniae upon the response to femoral fracture in the rat. *Am. J. Clin. Nutr.*, in press.
62. Kastello, M. D., G. A. Eddy, and R. W. Kuehne. 1976. A rhesus monkey model for the study of Bolivian hemorrhagic fever. *J. Infect. Dis.* 133:57-62.
63. Katz, M., W. R. Beisel, G. T. Keusch, L. J. Mata, R. Suskind, and E. R. Stiehm. 1976. Immune response of the malnourished child. A position paper of the Food and Nutrition Board of the National Research Council, National Academy of Sciences, Washington, D.C. 22 pp.
64. Kaufmann, R. L., C. F. Matson, and W. R. Beisel. 1976. Hypertriglyceridemia produced by endotoxin: role of impaired triglyceride disposal mechanisms. *J. Infect. Dis.* 133:548-555.
65. Kaufmann, R. L., C. F. Matson, A. H. Rowberg, and W. R. Beisel. 1976. Defective lipid disposal mechanisms during bacterial infection in rhesus monkeys. *Metabolism* 25:615-624.

66. Kenyon, R. H., and C. E. Pedersen, Jr., 1975. Preparation of Rocky Mountain spotted fever vaccine suitable for human immunization. *J. Clin. Microbiol.* 1:500-503.
67. Kenyon, R. H., L. St. C. Sammons, and C. E. Pedersen, Jr. 1975. Comparison of three Rocky Mountain spotted fever vaccines. *J. Clin. Microbiol.* 2:300-304.
68. Kenyon, R. H., P. G. Canonico, L. S. Sammons, L. R. Bagley, and C. E. Pedersen, Jr. 1976. Antibody response to Rocky Mountain spotted fever. *J. Clin. Microbiol.* 3:513-518.
69. Kirkpatrick, C. H., M. S. Ascher, and A. A. Gottlieb. 1976. Meeting report - Second International Workshop on Biological Properties and Clinical Applications of Transfer Factor. *Cell. Immunol.* 21:379-381.
70. Kishimoto, R. A., and J. S. Walker. 1976. Interaction between Coxiella burnetii and guinea pig peritoneal macrophages. *Infect. Immun.* 14: 416-421.
71. Larson, E. W., H. W. Young, and J. S. Walker. 1976. Aerosol evaluations of the DeVilbiss No. 40 and Vaponcfrin nebulizers. *Appl. Environ. Microbiol.* 31:150-151.
72. Larson, E. W., J. W. Dominik, A. H. Rowberg, and G. A. Higbee. 1976. Influenza virus population dynamics in the respiratory tract of experimentally infected mice. *Infect. Immun.* 13:438-447.
73. Leppla, S. H. 1976. Large-scale purification and characterization of the exotoxin of Pseudomonas aeruginosa. *Infect. Immun.* 14:1077-1086.
74. Levitt, N. J., H. V. Miller, and G. A. Eddy. 1976. Solid-phase radioimmunoassay for rapid detection and identification of Western equine encephalomyelitis virus. *J. Clin. Microbiol.* 4:382-383.
75. Liu, C. T., and G. A. Higbee. 1976. Determination of body surface area in the rhesus monkey. *J. Appl. Physiol.* 40:101-104.
76. Liu, C. T. 1976. Cardiovascular and renal functions in normal macaques. *Am. J. Vet. Res.* 37:969-974.
77. Liu, C. T., J. D. Helm, III, and W. R. Beisel. 1976. Cardiovascular and vomiting responses to a lethal intravenous dose of staphyloenterotoxin (A) in rhesus monkeys. *J. Med. Primatol.* 5: in press.
78. Liu, C. T., M. J. Griffin, and R. T. Faulkner. 1976. Effect of staphylococcal enterotoxin B (SEB) on body fluid compartments in conscious rhesus monkeys. *J. Med. Primatol.* 5: in press.
79. Long, G. G., J. L. Stookey, T. G. Terrell, and G. D. Whitney. 1975. Fibrous osteodystrophy in an opossum. *J. Wildlife Dis.* 11:221-223.
80. Long, G. G., T. G. Terrell, and J. L. Stookey. 1975. Hepatomas in a group of captive woodchucks. *J. Am. Vet. Med. Ass.* 167:589.

81. Long, G. G., J. D. White, and J. L. Stookey. 1975. Pneumocystis carinii infection in splenectomized owl monkeys. *J. Am. Vet. Med. Ass.* 167:651-654.
82. Long, G. G., J. R. Lichtenfels, and J. L. Stookey. 1976. Anatrichosoma cynamolgi (Nematoda: Trichinellida) in rhesus monkeys, Macaca mulatta. *J. Parasitol.* 62:111-115.
83. Luscri, B. J., O. M. Brand, and G. A. Eddy. 1975. The sensitivity of selected arenaviruses to human interferon. *Interferon Sci. Memo.* I-A191/1: 2-3.
84. Machotka, S. V., F. E. Chapple, III, and J. L. Stookey. 1975. Cerebral tuberculosis in a rhesus monkey. *J. Am. Vet. Med. Ass.* 167:648-650.
85. Mapes, C. A., and P. Z. Sobocinski. 1976. Multiple leukocytic factors that induce reactions characteristic of the inflammatory response. In Army Science Conference Proceedings, Vol. II. p. 405-419. Department of the Army, Washington.
86. Mapes, C. A., and P. Z. Sobocinski. 1976. Differentiation between endogenous pyrogen and leukocytic endogenous mediator. *Am. J. Physiol.* 231: in press.
87. Marker, S. C., and M. S. Ascher. 1976. Specific in vitro lymphocyte transformation with Venezuelan equine encephalitis virus. *Cell. Immunol.* 23:32-38.
88. McLeod, Jr., C. G., J. L. Stookey, G. A. Eddy, and S. K. Scott. 1976. Pathology of chronic Bolivian hemorrhagic fever in the rhesus monkey. *Am. J. Pathol.* 84:211-224.
89. McLeod, C. G., J. L. Stookey, and J. D. White. 1976. Intestinal Tyzzer's disease and spirochetosis in a guinea pig. *Vet. Pathol.* 13: in press.
90. McManus, A. T., and G. A. Eddy. 1975. Increased potency of killed VEE (TC-83) vaccine in mice previously infected with EEE virus. *Trans. R. Soc. Trop. Med. Hyg.* 69:172.
91. Metzger, J. F., A. D. Johnson, and L. Spero. 1975. Intrinsic and chemically produced microheterogeneity of Staphylococcus aureus enterotoxin type C. *Infect. Immun.* 12:93-97.
92. Moe, J. B., J. D. White, W. P. Czajkowski, and J. L. Stookey. 1975. Myxosarcoma in a young rhesus monkey. *Vet. Pathol.* 12:6-12.
93. Moe, J. B., P. G. Canonico, J. L. Stookey, M. C. Powanda, and G. L. Cockerell. 1975. Pathogenesis of tularemia in immune and nonimmune rats. *Am. J. Vet. Res.* 36:1505-1510.

94. Moe, J. B., G. L. Ruch, R. H. Kenyon, J. D. Burek, and J. L. Stookey. 1976. Pathology of experimental Rocky Mountain spotted fever in rhesus monkeys. *Vet. Pathol.* 13:69-77.
95. Moe, J. B., D. F. Mosher, R. H. Kenyon, J. D. White, J. L. Stookey, L. R. Bagley, and D. P. Fine. 1976. Functional and morphologic changes during experimental Rocky Mountain spotted fever in guinea pigs. *Lab. Invest.* 35:235-245.
96. Mosher, D. F. 1975. Cross-linking of cold-insoluble globulin by fibrin-stabilizing factor. *J. Biol. Chem.* 250:6614-6621.
97. Mosher, D. F., and D. A. Wing. 1976. Synthesis and secretion of α_2 -macroglobulin by cultured human fibroblasts. *J. Exp. Med.* 143:462-467.
98. Mosher, D. F. 1976. Action of fibrin-stabilizing factor on cold-insoluble globulin and α_2 -macroglobulin in clotting plasma. *J. Biol. Chem.* 251:1639-1645.
99. Mosher, D. F. 1976. Changes in plasma cold-insoluble globulin concentration during experimental Rocky Mountain spotted fever infection in rhesus monkeys. *Thromb. Res.* 9:37-45.
100. Neufeld, H. A., J. A. Pace, and F. E. White. 1976. The effect of bacterial infections on ketone concentrations in rat liver and blood and on free fatty acid concentrations in rat blood. *Metabolism* 25:877-884.
101. Neufeld, H. A., M. V. Kaminski, Jr., and R. W. Wannemacher, Jr. 1976. Effect of inflammatory and noninflammatory stress on ketone bodies and free fatty acids in rats. *Am. J. Clin. Nutr.*, in press.
102. Patrick, III, W. C., and W. R. Beisel. 1976. In-house peer review: an effective mechanism for research management. *Army Research and Development News Magazine* 17:4:19-20.
103. Pedersen, Jr., C. E., L. R. Bagley, R. H. Kenyon, L. S. Sammons, and G. T. Burger. 1975. Demonstration of Rickettsia rickettsii in the rhesus monkey by immune fluorescence microscopy. *J. Clin. Microbiol.* 2:121-125.
104. Pedersen, Jr., C. E. 1976. Preparation and testing of vaccines prepared from the envelopes of Venezuelan, Eastern, and Western equine encephalomyelitis viruses. *J. Clin. Microbiol.* 3:113-118.
105. Pekarek, R. S., and M. C. Powanda. 1976. Protein synthesis in zinc deficient rats during tularemia. *J. Nutr.* 106:905-912.
106. Petrella, V. J., and T. V. Zenser. 1976. Properties of cholera toxin- and NaF-stimulated adenylate cyclase from mouse thymocytes. *Biochim. Biophys. Acta* 421:237-245.
107. Pettit, G. W., M. R. Elwell, R. T. Faulkner, and K. A. Bostian. 1976. Oral fructose tolerance, gastric emptying and absorption: a compartmental model. *Arch. Int. Physiol. Biochim.* 84: in press.

108. Pettit, G. W., M. R. Elwell, and P. B. Jahrling. 1976. Endotoxemia in rabbits after intravenous injection of staphylococcal enterotoxin B. J. Infect. Dis. 134: in press.
109. Powanda, M. C., G. L. Cockerell, J. B. Moe, F. B. Abeles, R. S. Pekarek, and P. G. Canonico. 1975. Induced metabolic sequelae of tularemia in the rat: correlation with tissue damage. Am. J. Physiol. 229:479-483.
110. Powanda, M. C., R. E. Dinterman, R. W. Wannemacher, Jr., and W. R. Beisel. 1975. Tryptophan metabolism in relation to amino acid alterations during typhoid fever. Acta Vitaminol. Enzymol. 29:164-168.
111. Powanda, M. C., E. L. Henriksen, E. Ayala, and P. G. Canonico. 1976. Clofibrate-induced alterations in serum protein patterns. Biochem. Pharmacol. 25:785-788.
112. Powanda, M. C., R. H. Kenyon, and J. B. Moe. 1976. Alterations in plasma copper, zinc, amino acids, and seromucoid during Rocky Mountain spotted fever in guinea pigs. Proc. Soc. Exp. Biol. Med. 151:804-807.
113. Powanda, M. C., and P. G. Canonico. 1976. Protective effect of clofibrate against S. pneumoniae infection in rats. Proc. Soc. Exp. Biol. Med. 152:437-440.
114. Powanda, M. C. 1976. Changes in body balance of nitrogen and other key nutrients: description and underlying mechanisms. Am. J. Clin. Nutr., in press.
115. Rausch, P. G., and P. G. Canonico. 1975. Characterization of monkey peripheral neutrophil granules during infection. Infect. Immun. 12:687-693.
116. Rausch, P. G., and T. G. Moore. 1975. Granule enzymes of polymorphonuclear neutrophils: a phylogenetic comparison. Blood 46:913-919.
117. Rayfield, E. J., D. T. George, H. L. Eichner, and T. H. Hsu. 1975. L-Dopa stimulation of glucagon secretion in man. N. Engl. J. Med. 293:589-591.
118. Rayfield, E. J., R. T. Faulkner, and W. Czajkowski. 1976. Portal and peripheral vein insulin responses to intravenous glucose in the rhesus monkey. J. Lab. Clin. Med. 87:919-924.
119. Rayfield, E. J., L. Gorelkin, R. T. Curnow, and P. B. Jahrling. 1976. Virus-induced pancreatic disease by Venezuelan encephalitis virus. Alterations in glucose tolerance and insulin release. Diabetes 25:623-631.
120. Robinson, D. M., F. E. Cole, Jr., A. T. McManus, and C. E. Pedersen, Jr. 1976. Inactivated Mayaro vaccine produced in human diploid cell cultures. Mil. Med. 141:163-166.
121. Rosato, R. R., E. L. Stephen, and W. L. Pannier. 1976. Dose-response data for toxiferine dichloride in monkeys and guinea pigs. Toxicol. Appl. Pharmacol. 35:107-111.

122. Sammons, L. S., R. H. Kenyon, G. T. Burger, C. E. Pedersen, Jr., and R. O. Spertzel. 1976. Changes in blood serum constituents and hematologic values in Macaca mulatta with Rocky Mountain spotted fever. Am. J. Vet. Res. 37:725-731.
123. Sammons, L. S., R. H. Kenyon, and C. E. Pedersen, Jr. 1976. Effect of vaccination schedule on immune response of Macaca mulatta to cell culture-grown Rocky Mountain spotted fever vaccine. J. Clin. Microbiol. 4: 253-257.
124. Scott, G. H., and R. J. Sydiskis. 1976. Responses of mice immunized with influenza virus by aerosol and parenteral routes. Infect. Immun. 13:696-703.
125. Scott, G. H., and J. S. Walker. 1976. Immunoglobulin-bearing cells in lungs of mice infected with influenza virus. Infect. Immun. 13: 1525-1527.
126. Scott, S. K., P. C. Kosch, and D. E. Hilmas. 1976. Serum lactate dehydrogenase of normal, stressed, and yellow fever virus-infected rhesus monkeys. Lab. Anim. Sci. 26:436-442.
127. Scott, S. K., G. A. Eddy, and O. M. Brand. 1976. The African green monkey as a model for Bolivian hemorrhagic fever. Am. J. Trop. Med. Hyg. 25: in press.
128. Spero, L., J. F. Metzger, J. R. Warren, and B. Y. Griffin. 1975. Biological activity and complementation of the two peptides of staphylococcal enterotoxin B formed by limited tryptic hydrolysis. J. Biol. Chem. 250:5026-5032.
129. Spero, L., D. L. Leatherman, and W. H. Adler. 1975. Mitogenicity of formalinized toxoids of staphylococcal enterotoxin B. Infect. Immun. 12: 1018-1020.
130. Spero, L., B. Y. Griffin, J. L. Middlebrook, and J. F. Metzger. 1976. Effect of single and double peptide bond scission by trypsin on the structure and activity of staphylococcal enterotoxin C. J. Biol. Chem. 251: 5580-5588.
131. Sobocinski, P. Z., W. J. Canterbury, and K. H. Jurgens. 1976. Improved continuous-flow method for determination of total serum hexosamines. Clin. Chem. 22:1394-1396.
132. Steinhart, W. L., C. S. Hogeman, and M. C. Powanda. 1976. Inhibition of the production of infectious herpes simplex virus by clofibrate. Virology 70:241-243.
133. Stephen, E. L., J. W. Dominik, J. B. Moe, R. O. Spertzel, and J. S. Walker. 1975. Treatment of influenza infection of mice by using rimantadine hydrochlorides by the aerosol and intraperitoneal routes. Antimicrob. Agents Chemother. 8:154-158.

134. Stephen, E. L. 1976. First successful use of a chemical compound for the prophylaxis and treatment of a lethal, systemic, viral infection common to man and subhuman primates. In Army Science Conference Proceedings, Vol. III. p. 263-271. Department of the Army, Washington.
135. Stephen, E. L., J. W. Dominik, J. B. Moe, and J. S. Walker. 1976. Therapeutic effects of ribavirin given by the intraperitoneal or aerosol route against influenza virus infections in mice. Antimicrob. Agents Chemother. 10:549-554.
136. Summers, P. W. December 1975. Bibliography of Venezuelan Equine Encephalomyelitis (VEE), Supplement 4. U. S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD. 33 pp.
137. Thompson, W. L., F. B. Abeles, F. A. Beall, R. E. Dinterman, and R. W. Wannemacher, Jr. 1976. Influence of the adrenal glucocorticoids on the stimulation of synthesis of hepatic ribonucleic acid and plasma acute-phase globulins by leukocytic endogenous mediator. Biochem. J. 156:25-32.
138. Wachter, R. F., C. P. Briggs, J. D. Gangemi, and C. E. Pedersen, Jr. 1975. Changes in buoyant density relationships of two cell types of Coxiella burnetii phase I. Infect. Immun. 12:433-436.
139. Wachter, R. F., G. P. Briggs, and C. E. Pedersen, Jr. 1975. Precipitation of phase I antigen of Coxiella burnetii by sodium sulfite. Acta Virol. 19:500.
140. Wagner, F. S., C. G. McLeod, and G. A. Eddy. 1975. African green monkeys are susceptible to Machupo virus. Arthropod-Borne Virus Information Exchange 29:56.
141. Walker, J. S., E. L. Stephen, and R. O. Spertzel. 1976. Small-particle aerosols of antiviral compounds for the treatment of type A influenza pneumonia in mice. J. Infect. Dis. 133(Suppl.):A140-A144.
142. Wannemacher, Jr., R. W., A. S. Klainer, G. A. Higbee, and W. R. Beisel. 1976. Trace element changes during infections at various ages. In The Biomedical Role of Trace Elements in Aging (ed. J. M. Hsu, R. L. Davis and R. W. Neithamer). p. 175-187. Eckerd College Gerontology Center, St. Petersburg, FL.
143. Wannemacher, Jr., R. W., A. S. Klainer, R. E. Dinterman, and W. R. Beisel. 1976. The significance and mechanism of an increased serum phenylalanine-tyrosine ratio during infection. Am. J. Clin. Nutr. 29:997-1006.
144. Wannemacher, Jr., R. W., and R. E. Dinterman. 1976. Total body protein catabolism in starved and infected rats. Am. J. Clin. Nutr., in press.
145. Wannemacher, Jr., R. W. 1976. Key role of various individual amino acids in host response to infection. Am. J. Clin. Nutr., in press.

146. Warren, J. R., D. L. Leatherman, and J. F. Metzger. 1975. Evidence of cell-receptor activity in lymphocyte stimulation by staphylococcal enterotoxin. *J. Immunol.* 115:49-53.
147. Woodman, D. R., A. T. McManus, and G. A. Eddy. 1975. Extension of the mean time to death of mice with a lethal infection of Venezuelan equine encephalomyelitis virus by antithymocyte serum treatment. *Infect. Immun.* 12:1006-1011.
148. Woodward, T. E., W. R. Beisel, and R. D. Faulkner. 1976. Marylanders defeat Philadelphia: yellow fever updated. *Trans. Am. Clin. Climatol. Ass.* 87:69-101.
149. Woodward, T. E., C. E. Pedersen, Jr., C. N. Oster, L. R. Bagley, J. Romberger, and M. J. Snyder. Prompt confirmation of Rocky Mountain spotted fever: identification of rickettsiae in skin tissues. *J. Infect. Dis.* 134:297-301.
150. Zenser, T. V. 1975. Formation of adenosine 3',5'-monophosphate from adenosine in mouse thymocytes. *Biochim. Biophys. Acta* 404:203-213.
151. Zenser, T. V. 1976. Inhibition of cholera toxin-stimulated intestinal epithelial cell adenylate cyclase by adenosine analogs. *Proc. Soc. Exp. Biol. Med.* 152:126-129.

GLOSSARY

AkP	alkaline phosphatase
BHF	Bolivian hemorrhagic fever
CBC	complete blood count
CEC	chick embryo cell (culture)
CPE	cytopathic effect
DEC	duck embryo cell (culture)
ED ₅₀	median effective dose(s)
EEE	Eastern equine encephalitis (virus)
EMEM	Eagle's minimum essential medium
HA	hemagglutinins, hemagglutination
HI	hemagglutination inhibition
ID	intradermal(ly)
ID ₅₀	median infectious dose(s)
IM	intramuscular(ly)
IN	intranasal
IP	intraperitoneal(ly)
IV	intravenous (ly)
JE	Japanese encephalitis
LD ₅₀	median lethal dose(s)
LDH	lactic dehydrogenase
LEM	leukocytic endogenous mediator(s)
MA	microagglutination, microagglutinin
MMD	mass median diameter
MIPLD ₅₀	median infectious intraperitoneal lethal dose(s)
mRNA	messenger RNA
NDV	Newcastle disease virus
PBS	phosphate buffered saline
PFU	plaque forming unit(s)
P _i	inorganic phosphorus
pI	isoelectric point
PMN	polymorphonuclear leukocytes

PR ₅₀ or PR ₈₀	50% or 80% plaque reduction
PRNT	plaque reduction neutralization test
RBC	red blood cells
RES	reticuloendothelial system
RMSF	Rocky Mountain spotted fever
rRNA	ribosomal RNA
SC	subcutaneous (ly)
SDS	sodium dodecyl sulfate
SEA	staphylococcal enterotoxin A
SEB	staphylococcal enterotoxin B
SEC	staphylococcal enterotoxin C
SFV	Semliki Forest virus
SGOT	serum glutamic oxaloacetic transaminase
SGPT	serum glutamic pyruvic transaminase
SLE	St. Louis encephalitis (virus)
UV	ultraviolet
VEE	Venezuelan equine encephalomyelitis (virus)
VSV	vesicular stomatitis virus
WBC	white blood cells
WEE	Western equine encephalitis (virus)

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